Photobiologic, Toxicologic, and Pharmacologic Aspects of Psoralens

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Photobiologic, Toxicologic, and Pharmacologic Aspects of Psoralens

Proceedings
of a Conference
held at
Research Triangle Park,
North Carolina

March 1–3, 1982

Sponsored by:

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Scientific Editors:
Madhu A. Pathak, M.B., Ph.D.
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Coordinator

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Introduction[1]

A promising treatment for psoriasis is the use of psoralens and high-intensity longwave UV radiation. Extensive clinical studies, both in Europe and the United States, have indicated that this photochemotherapeutic approach is useful for disabling psoriasis, generalized psoriasis of the hands or feet, and several other diseases (e.g., vitiligo, mycosis fungoides, etc.), and it is preferable to the other currently available systemic agents which have demonstrated toxic effects on the bone marrow and gastrointestinal and central nervous systems. However, certain cooperative clinical studies have suggested that use of psoralens and UV irradiation may be associated with an increased risk of skin cancer in certain types of patients. At the request of the Food and Drug Administration and other government agencies, management personnel at the National Toxicology Program initiated a program to investigate the toxicologic properties of this treatment and to gather data which will aid in evaluating its effectiveness and safety. They also seek to understand the general toxicologic properties of UV light and chemical interactions.

On March 1–3, 1982, the National Toxicology Program sponsored this workshop on psoralens to 1) bring together scientific experts to discuss the relationships between structure and activity of various therapeutically useful psoralens and 2) review the toxicology data available with a view of suggesting ways to avoid or minimize certain toxicologic properties of this useful therapy. Ninety-six scientists, coming from 14 states and 7 foreign countries (France, West Germany, Italy, Finland, Austria, Denmark, and the United Kingdom), participated in the Conference. Among the topics discussed were photobiologic properties, pharmacokinetics, and pharmacodynamics of psoralens; mutagenicity; carcinogenic and immunologic aspects of psoralen and UV irradiation therapy; side effects of therapy; safety and therapeutic effectiveness; and analytic aspects of psoralen therapy.

This monograph includes the scientific presentations of this meeting as well as pertinent points which were highlighted during the discussion periods. We hope that it will serve as a summary document on the toxicology and therapeutic effectiveness of the combination therapy.

We want to thank Warren Posey for his help and assistance throughout this Conference, and also Dingle Associates, Mr. Ramsey Sa'di, Ms. Caroline Watler, and Ms. Cassie Harris for their contributions to its success.

Madhu A. Pathak, M.B., Ph.D.
Chairman

June K. Dunnick, Ph.D.
Coordinator

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[1] The receipt of some manuscripts was delayed.

SESSION I

Historical Aspects of Psoralens

Research and Development of Oral Psoralen and Longwave Radiation Photochemotherapy: 2000 B.C.–1982 A.D.[1]

Thomas B. Fitzpatrick *and* Madhu A. Pathak[2]

"Historic continuity with the past is not a duty, it is only a necessity."

Oliver Wendell Holmes, Jr.

In the past few decades, only a few treatments have made a real difference in the control of the most common skin diseases; acne, eczema, and psoriasis. In this small group of drugs we must include 1) topical corticosteroids for psoriasis and eczema; 2) oral or parenteral methotrexate for psoriasis; and 3) oral retinoids, such as isotretinoin, for cystic acne. Other retinoids, such as etretinate, are effective for certain types of psoriasis.

We are considering in this Symposium a newly introduced and highly effective treatment for psoriasis, i.e., the treatment known as oral PUVA photochemotherapy, in which an orally administered photoactive drug, methoxsalen (one of a series of furocoumarins known as psoralens) is followed in 1 to 2 hours by exposure to longwave UV radiation (>320 nm) or UVA; thus the acronym "PUVA" (psoralens plus UVA) was introduced. This new pharmacologic concept was termed "photochemotherapy," the combined use of electromagnetic energy and a drug, and, although first reported in 1974, it is in fact thousands of years old, having been used in Egypt and India since 1200–2000 B.C. Photochemotherapy for the common disfiguring disease, vitiligo, was practiced in the ancient world by physicians and herbalists who used boiled extracts of the fruits of certain umbelliferous plants, e.g., *Ammi majus* Linnaeus in Egypt (fig. 1) or the leguminous plant, *Psoralea corylifolia* L. in India. These preparations, which were made from seeds obtained from herbal stores (fig. 2), were either applied to the skin or ingested as an "infusion;" the patient then exposed the skin to the intense Egyptian or Indian sunlight. Vitiligo, a color imperfection of the skin, was and still is a major medical problem in India, inasmuch as it is regarded as "white leprosy." Victims are social outcasts who often cannot marry or get jobs and rarely may even resort to suicide.

It was Professor Abdel Monem El Mofty of the Department of Dermatology, Cairo University Medical School, Cairo, Egypt, who, in the early 1940s, first used crystalline methoxsalen (8-MOP) followed by exposure to sunlight in the treatment of vitiligo; it had just been isolated from *Ammi majus* L. by Egyptian pharmacologists. It was not until 27 years later, in 1974, that orally administered 8-MOP was first used in combination with a new high-intensity, artificial source of longwave UV radiation (320–400 nm), which is the action spectrum for psoralens. The new UVA light source was developed by Sylvania engineers in the United States in collaboration with investigators on the staff of the Dermatology Department of Harvard Medical School. This new UVA source was initially used in combination with either oral 8-MOP or 4,5′,8-trimethylpsoralen for the treatment of vitiligo by the Harvard dermatologists. Soon Parrish et al. (*1*) discovered that 8-MOP was also effective in psoriasis by using the paired-comparison technique (fig. 3A–D). They exposed one-half of the posterior trunk to a given dose of UVA and then administered oral 8-MOP, and 2 hours later they exposed the other half of the posterior trunk to the same dose of UVA. Repeated exposures (>20) in this manner resulted in a disappearance of the psoriatic lesions on the part treated with PUVA and no change in the lesions treated with UVA alone (fig. 3A–D). Using the same protocol, Wolff et al. (*2*) immediately confirmed these results in Vienna, Austria, in 1975.

During the years from 1974 to 1982, two teams comprising a dozen or more physician-investigators have collaborated to develop PUVA photochemotherapy at Harvard and in Austria. The Austrian group included W. Brenner, P. Fritsch, F. Gschnait, H. Hönigsmann, E. Jaschke, K. Konrad, and K. Wolff. Professor F. Greiter of Vienna, a physiologist, provided valuable assistance in the development of the application of PUVA photochemotherapy since 1974. Working on the Harvard team were T. B. Fitzpatrick, B. A. Gilchrest, E. Gonzalez, J. Melski, K. Momtaz-T, W. L. Morison, J. A. Parrish, M. A. Pathak, R. Stern, and L. Tanenbaum.

TIMING OF INTRODUCTION OF PSORALEN PHOTOCHEMOTHERAPY APPROPRIATE

After publication of reports on the effectiveness of oral PUVA in psoriasis in December 1974, widespread interest

ABBREVIATIONS: PUVA = psoralen plus UV radiation at 320–400 nm; 8-MOP = 8-methoxypsoralen; 5-MOP = 5-methoxypsoralen; MED = minimum erythema dose; J = joule(s); μW = microwatt(s); mW = milliwatt(s); UVB = UV radiation at 290–320 nm; 3-CP = 3-carbethoxypsoralen.

[1] Presented at the Conference on Photobiologic, Toxicologic, and Pharmacologic Aspects of Psoralens held in Research Triangle Park, North Carolina, March 1–3, 1982.

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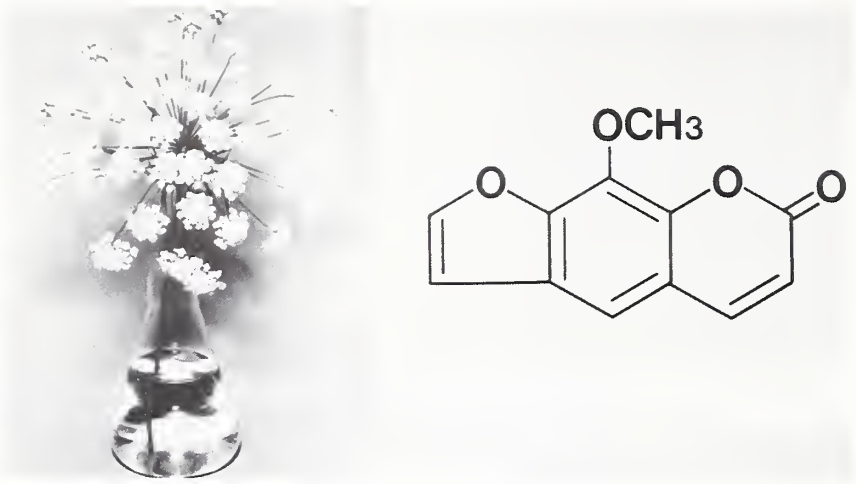

FIGURE 1.—*Ammi majus* L. and formula of major psoralen used for 35 yr, 8-MOP.

was shown in the use of this new therapy. The drug (8-MOP) was commercially available throughout the world, and some small electrical manufacturers in the United States and Europe had begun to make UVA radiation sources. However, this use of PUVA without experimental protocols was premature because initial problems in dosimetry of drug and of light required solutions obtainable only by large controlled clinical trials. Three multicenter clinical trials were conducted between 1975 and 1979 with controlled protocols and the accumulation of a large data base. Over 2,000 patients were studied in 25 centers in the United States (*3, 4*) and 2,995 in 17 major European centers (*5*). The European trial was coordinated by Professor K. Wolff in Vienna, Austria.

As a result of the accumulated experience begun in 1974 and the large multicenter clinical trials from 1975 to 1979, a detailed outline of the technique and side effects of PUVA was prepared in 1979. The Bureau of Radiological Health of the Food and Drug Administration then published specifications for the UVA irradiator in the *Federal Register,* and on May 20, 1982, after more than 7 years of extensive study, approved the use of PUVA for the treatment of psoriasis. The publication of a detailed package insert, prepared by Elder Pharmaceuticals (Bryan, Ohio) provided specific details of the PUVA technique. During the past 8 years, centers where this therapy is available have been established in all parts of the world, including Russia, Poland, and China.

The comprehensive clinical experience acquired in Europe and the results of large multicenter controlled trials in the United States and Europe have confirmed the high degree of efficacy of PUVA for the treatment of psoriasis and its short-term safety when administered by a standardized method and with a suitable UVA irradiator (*6*).

SIDE EFFECTS AND POTENTIAL RISKS OF PSORALEN PHOTOCHEMOTHERAPY: THREE DECADES OF CLINICAL EXPERIENCE

The discovery of PUVA stimulated research into all aspects of psoralens; for example, 790 articles and publications appeared on psoralens in 1978 and 1979 alone. Yet

FIGURE 2.—Prof. A. M. El Mofty in front of the herbal store in the *suq* in Cairo. This photograph was taken in 1976 and this store is still a source of the seeds which Fahmy and Abu-Shady obtained there in 1947 and from which they isolated 8-MOP, the first psoralen widely used in clinical studies.

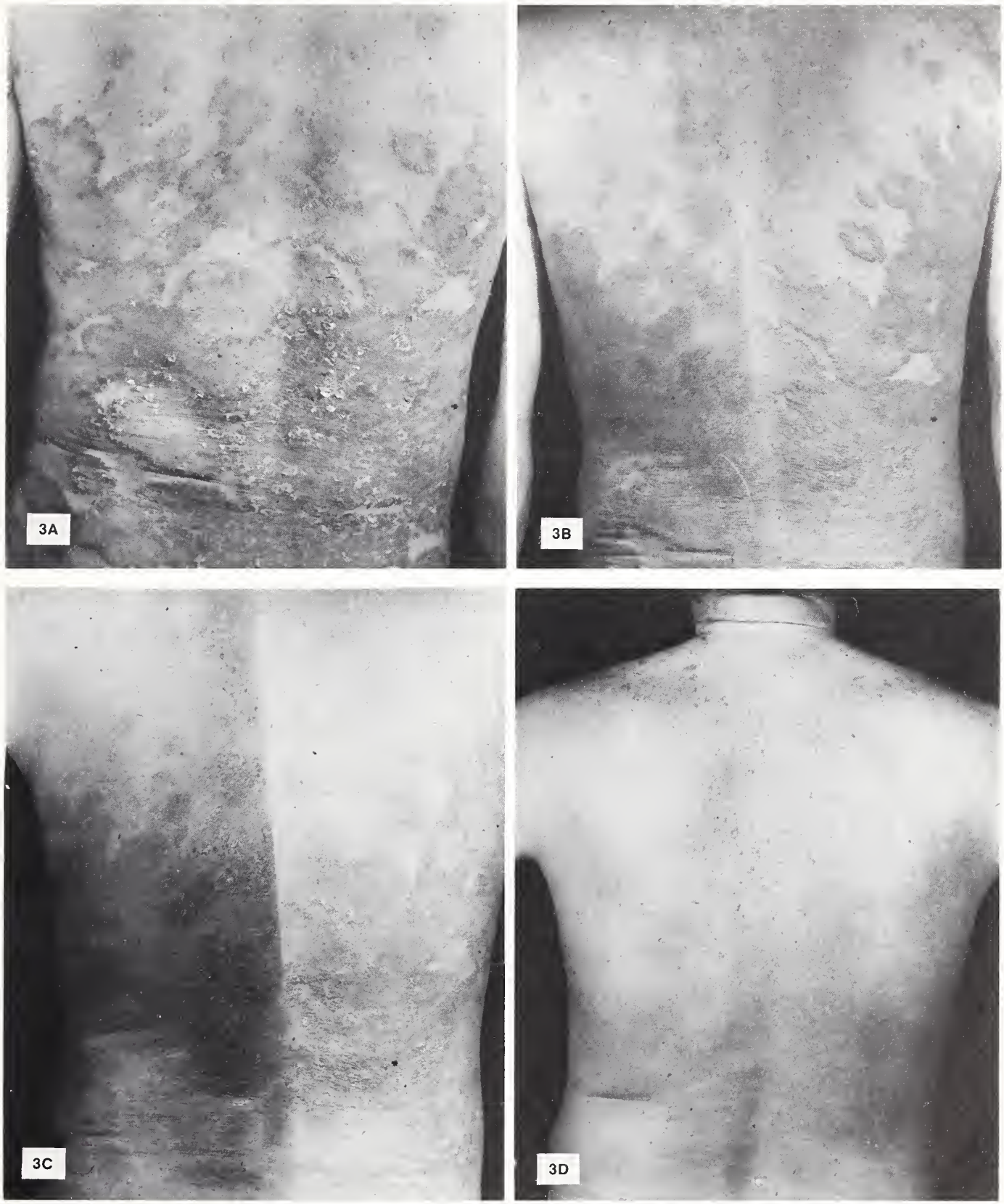

FIGURE 3.—Generalized psoriasis in the first patient ever treated with oral PUVA photochemotherapy on January 6, 1974 in Boston at the Massachusetts General Hospital. Patient was 41-year-old male with intractable disabling psoriasis. A) Before treatment; B) after 4 exposures to PUVA. *Note* flattening of lesions and pigmentation on the *left side* that was with PUVA. No change on the *right side* treated with UVA alone. C) After 7 treatments with PUVA and UVA. *Note* marked flattening of the lesions on the *left side* treated with PUVA alone. D) After 20 PUVA exposures to both sides; psoriasis has completely disappeared.

this burst of research can most accurately be termed a renaissance, as psoralens had been used for the treatment of vitiligo for over 30 years. A monograph on the chemistry, pharmacology, and toxicity of psoralens was published 23 years ago (*7*).

Several thousand patients have been treated for vitiligo with oral psoralens and with sunlight as the source of UVA since their introduction in Egypt in 1947. During this 35-year clinical experience (*8*), virtually no side effects have been reported; however, infrequent side effects or common disorders, such as skin cancer in whites, may have escaped attention because no prospective studies were conducted nor were any detailed follow-up reports published. Moreover, the "new" PUVA involved the interaction of oral psoralens with an artificial high-intensity source emitting UVA radiation.

Although the exposures to UVA when lamps are used are shorter than when sunlight is the source, a larger number and greater frequency of treatments and maintenance therapy may be continued throughout the year. These factors could change the incidence and type of long-term side effects; experience from the large clinical trials has shown that acute side effects can be avoided by careful dosimetry of drug and light. In the United States and Europe, over 5,000 patients have been studied prospectively (*9*). The first 1,300 patients comprising the initial 16-center trial who were treated during the 1970s are still being followed in the respective centers with physical examinations, serial eye examinations, and various laboratory tests.

STEPPING STONES IN THE DEVELOPMENT OF PSORALEN PHOTOCHEMOTHERAPY

We selected a number of the research contributions that form the basis for PUVA photochemotherapy. These facts, developed by both clinical and basic science investigators, are presented in summary form below in chronologic sequence with references provided.

The story of the development of PUVA photochemotherapy begins with the first identification in 1938 of psoralens as the basis for photosensitization in certain plants and subsequently by the isolation of 3 psoralens from plants in 1947. A year later, the first clinical use of 8-MOP as an oral photoactive chemical for the treatment of vitiligo was made. Eight years later, dose–response studies were done that demonstrated photosensitization in humans by orally administered psoralens followed by exposure to sunlight. In 1957, it was clear that psoralens were not photodynamic agents because their action did not require molecular oxygen. At this time, psoralens were shown to be activated by UVA. The lethal photosensitization of bacteria by psoralens and UVA was believed based on the capacity of psoralens to bind to DNA in the presence of UVA. Linear psoralens can form cross-links (bifunctional adducts) with 2 strands of DNA, whereas other nonlinear psoralens (isopsoralens) form only monofunctional adducts. Clinical studies of the action of psoralens plus artificial UVA on diseases other than vitiligo were begun when 8-MOP, applied topically to the skin, was first used in 1972 to induce remission of plaques of psoriasis. Two years later, it was reported that oral 8-MOP in combination with newly developed, high-intensity UVA irradiators induced a remission in 20 patients with psoriasis. This combination of drug and photons was termed "photochemotherapy" (specifically, "PUVA photochemotherapy"). Several thousand patients with psoriasis were then treated in multicenter clinical trials in the United States and Europe from 1975 to 1979. These clinical trials conducted in university centers provided a large data base from which evolved a treatment regimen for oral PUVA photochemotherapy. With careful monitoring of the initial 1,300 patients, investigators detected a small incidence of carcinoma (2.5%), first reported in 1979. Without a control group of patients with psoriasis who received treatment other than PUVA, they could not prove the photocarcinogenicity of oral PUVA photochemotherapy in humans. Nevertheless, the reversal of the ratio of basal cell carcinoma to squamous cell carcinoma in the population of patients treated with PUVA was highly suggestive that this therapy was photocarcinogenic.

The selected studies are as follows:

1) Photosensitization of skin by plants as related to the presence of furocoumarins was first described by Kuske (*10*) in 1938. He identified natural furocoumarins in plants as photosensitizers and isolated bergapten (5-MOP) from the oil of bergamot. This Swiss dermatologist's pioneer study was probably the first indication that furocoumarins were photoactive agents.
2) In 1947, Fahmy and his student, Abu-Shady, reported (*11*) the isolation of 8-MOP from *Ammi majus* L. They prepared an alcoholic extract of powdered fruit of *Ammi majus* L. and gave it in capsule form to patients who were then exposed to Egyptian sunlight for 30 minutes (~6 J UVA/cm^2). Patients developed gastrointestinal symptoms, but those who could tolerate the extract showed repigmentation of vitiligo macules. Later, crystalline 8-MOP was isolated from this plant, and a dose of 50 mg was given to patients. [Professor Fahmy and Abu-Shady obtained their seeds at an herb store in the bazaar in Cairo. This store is still in existence, and one can obtain the seeds of *Ammi majus* L. complete with an instruction sheet for the preparation of an infusion. This is then ingested and the patient exposes his/her skin to the sun. This treatment for vitiligo was among the first for psoralen use until the mid-1970s.]
3) El Mofty (*12*), a leading Egyptian dermatologist, pioneered the treatment of vitiligo with a crystalline compound, 8-MOP. He reported his findings on the effectiveness of 1) topical 8-MOP plus exposure to sunlight, 2) an oral dose of 40–50 mg 8-MOP plus exposure to sunlight, and 3) a combination of the topical and oral treatment in the repigmentation of vitiliginous macules. Although El Mofty had no controls, his study has some validity because patients with vitiligo rarely spontaneously repigment; furthermore, exposure to sunlight alone is ineffective, as El Mofty detected.
4) Clinical and experimental studies were performed by Lerner et al. (*13*) who also used 8-MOP for vitiligo.

They reported a dose of 400–600 mg 8-MOP was lethal to 50% of the animals treated. In the uncontrolled clinical study, 3 albinos given 30 mg daily with random exposures to sunlight gave testimonial evidence of increased tolerance to sunlight. That the MED increased in the 3 patients suggested a decrease in sensitivity to sun, even though they did not tan. This report of the first use of oral psoralens in the United States confirmed the clinical efficacy in the treatment of vitiligo and the safety of 8-MOP in man over a period of several months. However, whether albinos develop increased tolerance when psoralens and exposure to the sun are used has not been established.

5) Fitzpatrick and associates (*14*) conducted dose–response studies in 1955 in which 63 volunteers were randomized in double-blind and cross-over design trials in Oregon, Idaho, and Arizona. Oral administration of 50 mg 8-MOP before exposure to graduated amounts of sunlight demonstrated augmented cutaneous responses (erythema, edema) 44 hours after exposure and markedly increased tanning in 1 week. After oral ingestion of 75 mg followed by hourly exposures to sunlight, blistering of the exposed site was observed 2 hours after the drug was taken. Results of this controlled study indicated that oral psoralen plus exposure to sunlight was phototoxic and increased the facultative melanin pigmentation considerably. Also established was the fact that the maximum phototoxic effect occurs 1.5–2.0 hours after oral administration.
6) Musajo (*15*) first described the mutagenic properties of 5 furocoumarins found in onion root tips. At 5×10^{-5} *M*, 5-MOP induced mitosis with chromosome mutations. Musajo, after experiencing the photosensitizing effect of bergamot oil on his skin while vacationing in Calabria, Italy, began in 1955 a 25-year organized research study of furocoumarins. He demonstrated the structure–activity relationships and the mutagenic properties of some linear psoralens. His laboratory at the University of Padua is still the leading center for research on the chemistry of psoralens.
7) Subsequently in 1959, Oginsky and her co-workers (*16*) reported the killing of bacteria by psoralens and UVA. These investigators demonstrated that the lethal photosensitization of bacteria by psoralens plus UVA was not an oxygen-dependent photodynamic event. They also indicated that the active wavelengths were in the range of 320–400 nm.
8) After daily exposures to 43 J UVA/cm^2 for 6 weeks, 8-MOP, given to each mouse in ip doses of 0.4 mg/day, was carcinogenic to the skin of albino mice (*17*). However, the light source also contained a small fraction of UVB. Griffin's study (*17*) presents certain problems: 1) The radiant energy to which the mice were exposed contained 290–400 nm, i.e., both UVA and UVB, and we know that UVB is carcinogenic when administered without psoralens; 2) the mice received the equivalent of 700 mg/70 kg: seventeen times the human therapeutic dose; and 3) they were exposed to large amounts of UVA. Nevertheless, when given ip and followed by UVA (but no UVB), 8-MOP is carcinogenic in mice.
9) The feasibility of increased erythema and pigmentation following oral 8-MOP and low-intensity UVA was first demonstrated by Stegmaier (*18*) in 1959. Patients were given oral doses of 50 mg 8-MOP and then were exposed to 7 daily UVA exposures from 3 "blacklight" 40-W fluorescent lamps for 30 minutes; the total dose amounted to 3.95 J/cm^2. [El Mofty (*12*) had shown previously that topical 8-MOP was phototoxic at much shorter exposures to sunlight than when 8-MOP is taken orally.]
10) Also in 1959, Imbrie et al. (*19*) established that oral 8-MOP and a single exposure to sunlight could alter the skin tolerance to UVB (probably by increased facultative melanogenesis and increased thickness of the epidermis), so that double the dose of UVB was required to induce an erythema. Thus 8-MOP plus sunlight increased the threshold for the MED to UVB. Increased tolerance of exposed skin to subsequent challenge of 170 μW UVB/cm^2 obtained from a 175-W sunlamp was observed in 8 subjects who were exposed to 30 minutes of sunlight 2 hours after ingestion of 30 mg 8-MOP. Imbrie and co-workers noted that twice as much UVB was required to produce erythema on skin areas of the subjects who received the psoralen plus sunlight compared with those given a placebo and sun exposure.
11) Buck and associates (*20*) localized the action spectrum of 8-MOP to 360 nm after noting that delayed erythema (36 hr) resulted after humans were exposed to 360 nm radiant energy. Their studies were extended by other investigators (*21*) who found that the action spectrum is in the shorter wavelength region, i.e., as short as 320 nm, with the peak wavelength at 330–340 nm.
12) A mechanism of skin photosensitization based on the production of singlet excited state and metastable triplet state of psoralens was reported by Pathak (*21*) in 1961. Pathak demonstrated the existence of reactive singlet and triplet states of psoralens. With supporting experimental evidence, he proposed that the mechanism of biologic photosensitization evoked by psoralens involved excitation of the molecule to a singlet excited state, followed by a transition to a metastable triplet state. Psoralens in the triplet state were shown to generate free radicals, which were believed to evoke biologic photosensitization.
13) The first evidence of photobinding of furocoumarin molecules to DNA was provided by Musajo et al. (*22*). By examining the modification of the fluorescence spectrum of the psoralen during UV irradiation in the presence of DNA, RNA, nucleosides, and the purine or pyrimidine bases, they concluded that the furocoumarin molecule was covalently photobound to DNA.
14) Additional work reported by Musajo and others (*23*) in 1967 indicated that PUVA inhibits the tumor-transmitting capacity of ascites tumor cells. They demonstrated the inactivation of these cells by

irradiating them in the presence of psoralen, 8-MOP, and 5-MOP.

15) By 1968, it was evident that psoralens did photoreact with DNA. Dall'Acqua and associates (*24*) showed that psoralens formed 2 types of photoadducts at the 3,4- and the 4',5'-positions in the psoralen molecule. When a frozen aqueous solution in which the psoralen and thymine molecules were mobilized in a matrix was irradiated, both 3,4- and 4',5'-photoadducts were obtained. This involved the 3,4- or 4',5'-double bond of the drug and the 5,6-double bond of the pyrimidine.

16) Pathak and Krämer (*25*) established the in vivo photoreaction of psoralen with DNA in 1969. Application of tritiated trioxsalen to the skin and irradiation with 365 nm UVA and subsequent extraction of DNA, RNA, and proteins from the irradiated skin showed in vivo photoconjugation of psoralen with DNA and RNA. However, photoconjugation of psoralens with epidermal protein fractions was minimal. Fluorescent and nonfluorescent adducts were formed as well. Whether inhibition of DNA synthesis is the only basis for the therapeutic effect of PUVA on psoriasis has not been established.

17) Soon after these observations, investigators in the United States and Italy simultaneously demonstrated intercalation of psoralen and subsequent formation of interstrand cross-links between psoralen and pyrimidine bases of 2 strands of DNA. The two most important published works on the formation of cross-links by psoralens and UVA in DNA are that of Cole (*26*) and Dall'Acqua et al. (*27*).

18) Mortazawi (*28*), an Iranian dermatologist working with Oberste-Lehn in Wuppertal, Federal Republic of Germany, reported on the induction of a remission in psoriasis after topical application of 8-MOP (0.15%) and 20 exposures to UVA from a 16-bulb light source. Tronnier and Schule (*29*), using essentially the same protocol, made the same observations.

19) In their studies of the synthesis of the nucleic acids in Ehrlich ascites tumor cells by irradiation in the presence of skin-photosensitizing and nonphotosensitizing furocoumarins, Bordin and associates (*30*) detected strong inhibition of DNA and RNA synthesis. The most potent compound was 8-MOP.

20) The first controlled study of oral 8-MOP and high-intensity UVA in the treatment of psoriasis was reported in 1974 by the Harvard group (*1*). This report introduced carefully monitored dosimetry with a new high-intensity UVA source and the term "photochemotherapy." Paired comparison studies were conducted on 16 patients with generalized psoriasis. Conventional doses of UVB were compared with high-intensity UVA alone (UVB was excluded with the use of Mylar). Each patient was given 0.6 mg 8-MOP/kg orally and exposed to the radiant energy thrice weekly. Initial exposures of 2.4 to 4.8 J/cm^2 were increased at each exposure time by 0.34 to 0.68 J/cm^2. By the end of a week of treatment, the side treated with PUVA was markedly more improved than the side that received UVB. Complete clearing on the PUVA-treated side occurred in 12–18 treatments. Using the same protocol and light system, K. Wolff noted identical results in 29 of 30 patients treated in Vienna, Austria.

21) The stimulus for GTE-Sylvania to build a high-intensity UVA source was the need for an all-year treatment for vitiligo. Levin, a physicist, and Parrish, a dermatologist, collaborated in the design of the first lighting units that made PUVA therapy a practical reality (*31*). Initial exposure times (5 min) were reduced from the conventional blacklight sources (40 min) used previously. This lighting system had 48 horizontal lamps aligned in a parallel array on a 4.4-cm center. Plane reflectors of aluminum were located vertically and were perpendicular to the lamps. Only small variations in irradiance occurred over an adequate working distance of about 40.0 cm. Irradiance was 9 mW/cm^2 for the 320- to 380-nm band with an average exposure time of 5–10 minutes compared with 0.7 mW/cm^2 in the 320- to 380-nm band for 12 blacklight fluorescent lamps (40-W, 48-inch) aligned horizontally in a parallel array on 15-cm centers with aluminum plane reflectors. This latter system required a minimum exposure time to induce melanogenesis and phototoxicity (following oral psoralen).

22) The treatment of mycosis fungoides with PUVA was found effective in a clinical study conducted by Gilchrest et al. (*32*). Of 11 patients, 4 were erythrodermic and 7 were in plaque stage; 8 cleared to less than 5% involvement and of 3 erythrodermic, 2 improved and 1 did not. Follow-up results 700–1,000 days afterward of the 8 patients who experienced clearing of their disease revealed that 5 were on maintenance therapy and were controlled, and 3, who stopped receiving PUVA therapy for reasons not related to complications of treatment, had recurrence of mycosis fungoides. Inasmuch as sunlight had been known to induce remission in this skin neoplasm, PUVA was a reasonable mode of therapy. Since this article was published, several reports confirmed the effectiveness of PUVA in the early stages of mycosis fungoides. The treatment is only palliative, as are all available treatments for this disease (e.g., electron beam therapy, topical nitrogen mustard).

23) The results establishing the efficacy of PUVA in the clearing and remission of psoriasis were reported in 1977 by Melski and co-workers (*3*). This report of cooperative trials conducted in 16 academic dermatology departments in the United States proved that effective PUVA photochemotherapy requires close attention to detail and careful monitoring. Although all the centers used the same protocol, light source, and medication, the percentages of patients who showed clearing varied from center to center; some centers observed a 90% clearing, whereas others stated they had 40%. The centers

have 169 patients still under treatment for clearing psoriasis. The results are tabulated as follows:

Patient status	No. of patients	Percentage of patients
Psoriasis cleared		
<30 treatments	787	69
>30 "	218	19
Dropped out of treatment		
Failed to clear	33	3
PUVA complications	14	1
Other complications	18	2
Other reasons	69	6
Total	1,139	100

24) Between 1975 and 1977, photochemotherapy of psoriasis with oral PUVA became extremely popular. Simultaneously, misleading anecdotal articles started appearing in print, such as that by Reed and associates (*33*) of a 14-year-old girl with xeroderma pigmentosum (DeSanctis-Cacchione type) who died of pneumonia. The child had had many nonmelanoma skin cancers (basal cell and squamous cell carcinomas) and keratocanthomas before she was 6 years old. At age 13, she was treated for 1 month with trioxsalen (no dose stated) "with very poor results, apparently with a greater number of skin lesions." This report has no scientific basis because it was completely uncontrolled. It is impossible for one to observe a change in the "number of skin cancers" without a control period. Furthermore, the medication was only administered for 1 month. It is unfortunate that this misleading published report is frequently cited as one indicating the carcinogenic action of psoralens in man.

25) Since the time 8-MOP was found to be a phototoxic and potentially carcinogenic agent, investigators have been exploring the usefulness of nonphototoxic and noncarcinogenic psoralens. A first report on a noncarcinogenic yet clinically effective psoralen appeared in 1978, the work of Dubertret and coworkers (*34*). They determined that 3-CP formed only monofunctional adducts in yeast. Monofunctional adducts are more easily repaired than are cross-links formed by 8-MOP. Topical application of 15 mg 3-CP/cm^2 to ears of mice before they were irradiated at a dose rate of 28 $J \cdot cm^{-2} \cdot second^{-1}$ resulted in no tumor formation after 196 applications compared with development of tumors in 90% when 8-MOP was used topically. Intraperitoneal administration of 0.4 mg/Swiss mouse was followed by UVA exposure at 1.68×10^4 J/cm^2. Thirty-six injections of 0.4 mg 3-CP/mouse followed by UVA produced neither tumors nor toxicity, but 8 mice given 8-MOP in the same dosage developed the same percentage of tumors as with topical application. In the clinical studies reported by these authors (*34*), 4 of 10 patients had good therapeutic results with slow resolution from an average of 33 treatments and a mean total dose of 417 J/cm^2. Although 3-CP is noncarcinogenic, it is only weakly effective in treating psoriasis when applied topically and followed by exposure to UVA. The clinical studies were inconclusive; perhaps other derivatives of this type may also be carcinogenic and clinically effective, when given topically or orally.

26) The efficacy of PUVA in maintenance of remission in psoriasis was carefully investigated by Wolff et al. (*35*) who demonstrated that a period of maintenance for 60 days provides for a prolonged remission (over 1 yr). Relapses gradually drop as a function of time but occur during the first 2 months of therapy. With no maintenance treatment, Wolff and associates determined that 11% of their patients were free of disease after 56 weeks; if such therapy was provided for 2–3 months after clearing, more than 66% were still in remission after 33 weeks. They concluded that by discontinuing treatment after 2 months, one could sort out the subset of patients who tend to relapse. Some pertinent data of this study conducted in Innsbruck and Vienna, Austria, are as follows:

No. of patients	572
No. of patients cleared	534 (93%)
No. of exposures to UVA required for clearing	14.7 ± 8.3
Duration of treatment, days	30.4 ± 26.6
Total UVA dose, J/cm^2	78.7 ± 88.7
No. of treatment failures	37 (6.5%)

27) The increased incidence of squamous cell carcinoma in certain susceptible patients with psoriasis who were treated with PUVA was documented by careful observation at 16 academic and medical centers in the United States (*9*). In humans, an increased relative frequency of squamous cell carcinoma in areas not habitually exposed (trunk and lower extremities) to sunlight was observed. The tumors, which occurred most frequently in patients with previous exposure to ionizing radiation, were nonaggressive, and no metastases were observed. This report emphasized a reversal of the ratio of basal cell carcinoma to squamous cell carcinoma, i.e., a greater number of squamous than basal cell carcinomas were observed in patients treated with PUVA. Also, the former type of neoplasms was reportedly seen in body sites not normally exposed to sunlight. The overall incidence in 25 months was 2.5% (30 tumors in 1,177 patients). The magnitude of this problem is uncertain because some groups with large patient populations and careful follow-up did not experience an increased incidence of skin carcinomas. Stern et al. (*9*) showed only an increased incidence of squamous cell carcinoma.

28) A report on the cooperative study among 18 European centers on the efficacy of PUVA in remission of psoriasis was published in 1981 (*5*). This multicenter European trial confirmed results of the studies conducted in the United States. Maintenance

with PUVA had little or no effect on the duration of remission. The data are tabulated as follows:

Parameter	No.	Percent of clearance	Percent of patients cleared
Total patients	3,136		
Cleared	2,785	90–100	88.8
Improved	179	50–90	5.7
Moderately improved	91	20–50	2.9
Unchanged or minimally improved	53	0–20	1.7
Treatment failures	28		0.9
Exposures required for clearing, mean	19.0		
Duration of treatment required for clearing, mean No. of wk	5.7		
Total cumulative dose required for clearing, mean No. of J/cm^2	103.3		

CONCLUSIONS

The development of oral PUVA photochemotherapy has evolved from the efforts of several dozen clinician-investigators in the United States and Europe who conducted the tedious clinical trials in over 7,500 patients. This data base is the standard of reference for all claims of efficacy and acute toxicity treatment of humans with oral 8-MOP and UVA. Therapy with PUVA is effective in an increasing number of cutaneous disorders including a neoplasm that arises in the skin, i.e., T-cell lymphoma or mycosis fungoides. In some disorders, PUVA is the only effective treatment available, in others, it is a reasonable option, and in others, it is safer than corticosteroids.

TABLE 1.—*Treatment responses with oral PUVA photochemotherapy*

Type of disease	No. of treatments required	Patient response
Psoriasis	12–40	Clearing in 90%; maintenance therapy required in 30%
Vitiligo	100–200	Excellent improvement on the head and neck in over 70%; incomplete responses in 30%
Mycosis fungoides	20–60	Prolonged clearing in early stages of disease
Polymorphous light eruption	10–40	Improvement in over 90%
Palmar, plantar vesicular dermatitis	20–60	" " " "
Eczema	20–60	Initial clearing in all of 15; aggressive therapy and maintenance required

For repigmentation in patients with vitiligo (*36*); clearing (*37*) of psoriasis of the palms and soles (etretinate, though effective, is not available in the United States); vesicular dermatitis ("dyshidrotic") eczema (*38*) of the hands and feet (PUVA is also safer than systemic corticosteroids); and for actinic reticuloid (*39*), PUVA is the only effective treatment available.

Clinical experience with carefully controlled studies indicates that PUVA is also good for treating disabling psoriasis (*6*), compared with UVB or methotrexate; polymorphous light eruption (*40*); mycosis fungoides (*41*) in its early stages, compared with topical nitrogen mustard; and solar urticaria in preference to UVA alone. These controlled clinical studies have also revealed that PUVA is an alternative therapy to corticosteroids in generalized plaque or papular psoriasis (*6*) because topical steroids are contraindicated in generalized psoriasis; chronic atopic eczematous dermatitis (*42*) rather than prolonged topical corticosteroids; and pustular psoriasis (von Zumbusch) as compared with systemic corticosteroids or etretinate (*43*).

Risk–benefit decisions will vary with the disease being treated (table 1). If 5 exposures to PUVA each year allow a patient with polymorphous light eruption to resume a normal life, this is a small risk for considerable benefit. Fifty treatments to induce clinical and histologic remission in the early stages of mycosis fungoides is a warrantable alternative to other therapy options. In many patients with disabling psoriasis, PUVA is more effective than the Goeckermann (UVB plus tar) or the Parrish regimen (UVB plus emollients). It is a logical alternative to methotrexate, and in many patients little maintenance therapy is required. Hundreds of exposures to PUVA for a period of years for treatment of small areas of psoriasis is not justified. For certain disorders, such as disabling psoriasis and mycosis fungoides, the choice lies not between risk and safety, but among alternate courses of action, none of which is absolutely safe. In the final analysis, perhaps the development of the concept of photochemotherapy as a pharmacologic principle was as important as the development of PUVA itself.

REFERENCES

(*1*) PARRISH JA, FITZPATRICK TB, TANENBAUM L, et al: Photochemotherapy of psoriasis with oral methoxsalen and longwave ultraviolet light. N Engl J Med 291:1207–1211, 1974

(*2*) WOLFF K, HÖNIGSMANN H, GSCHNAIT F, et al: Photochemotherapie bei Psoriasis. Klinische Erfahrungen bei 152 Patienten. Dtsch Med Wochenschr 100:2471–2477, 1975

(*3*) MELSKI JW, TANENBAUM L, PARRISH JA, et al: Oral methoxsalen photochemotherapy for the treatment of psoriasis: A cooperative clinical trial. J Invest Dermatol 68:328–335, 1977

(*4*) ROENIGK HH JR, MARTIN JS: Photochemotherapy for psoriasis. Arch Dermatol 113:1667–1670, 1977

(*5*) HENSELER T, WOLFF K, HÖNIGSMANN H, et al: Oral 8-methoxypsoralen photochemotherapy of psoriasis. The European PUVA Study: A cooperative study among 18 European centres. Lancet 1:853–857, 1981

(*6*) WOLFF K, HÖNIGSMANN H: Clinical aspects of photochemotherapy. Pharm Ther 12:381–418, 1981

(7) KAN NM (ed): Psoralens and radiant energy. J Invest Dermatol 32(Suppl):132–383, 1959

(8) PARRISH JA, STERN RS, FITZPATRICK TB: Evaluation of PUVA–1980: Its basic nature and toxicity. *In* Dermatology Update—Reviews for Physicians (Moschella SL, ed). New York: Elsevier, 1982, pp 313–337

(9) STERN RS, THIBODEAU LA, KLEINERMAN RA, et al: Risk of cutaneous carcinoma in patients treated with oral methoxsalen photochemotherapy for psoriasis. N Engl J Med 300:809–813, 1979

(10) KUSKE H: Experimentelle Untersuchunger zur Photosensiliaierung der Haut durch pflanzliche Wirkstoffe. Lichtsensibilisierung durch Furocoumarine als Ursache vershiedener phytogener Dermatosen. Arch Dermatol Syph 178:112–123, 1938

(11) FAHMY IR, ABU-SHADY H: *Ammi majus* Linn: Pharmacological study and isolation of a crystalline constituent, ammoidin. Q J Pharm Pharmacol 20:281–291, 1947

(12) EL MOFTY AM: A preliminary clinical report on the treatment of leukoderma with *Ammi majus* Linn. J R Egypt Med Assoc 31:651–665, 1948

(13) LERNER AB, DENTON CR, FITZPATRICK TB: Clinical and experimental studies with 8-methoxypsoralen in vitiligo. J Invest Dermatol 20:299–314, 1953

(14) FITZPATRICK TB, HOPKINS CE, BLICKENSTAFF DD, et al: Augmented pigmentation and other responses of normal human skin to solar radiation following oral administration of 8-methoxypsoralen. J Invest Dermatol 25:187–190, 1955

(15) MUSAJO L: Interessanti proprietà delle furocoumarine naturali. Farmaco [Sci] 10:539–558, 1955

(16) OGINSKY EL, GREEN GS, GRIFFITH DG, et al: Lethal photosensitization of bacteria with 8-methoxypsoralen to long-wave ultraviolet radiation. J Bacteriol 78:821–833, 1959

(17) GRIFFIN AC: Methoxsalen in ultraviolet carcinogenesis in the mouse. J Invest Dermatol 32:367–372, 1959

(18) STEGMAIER OC: The use of methoxsalen in sun tanning. J Invest Dermatol 32:345–349, 1959

(19) IMBRIE JD, DANIELS F, BERGERON L, et al: Increased erythema threshold six weeks after a single exposure to sunlight plus oral methoxsalen. J Invest Dermatol 32: 331–337, 1959

(20) BUCK HW, MAGNUS IA, PORTER AD: The action spectrum of 8-methoxypsoralen for erythema in human skin. Br J Dermatol 72:249–255, 1960

(21) PATHAK MA: Mechanism of psoralen photosensitization and in vivo biological action spectrum of 8-methoxypsoralen. J Invest Dermatol 37:397–406, 1961

(22) MUSAJO L, RODIGHIERO G, DALL'ACQUA F: Evidences of a photoreaction of the photosensitizing furocoumarins with DNA and with pyrimidine nucleosides and nucleotides. Experientia 21:24–25, 1965

(23) MUSAJO L, VISENTINI P, BACCICHETTI F, et al: Photoinactivation of Ehrlich ascites tumor cells obtained with skin-photosensitizing furocoumarins. Experientia 23:335–336, 1967

(24) DALL'ACQUA F, MARCIANI S, BORDIN F, et al: Studies on the photoreaction (365 nm) between psoralen and thymine. Ric Sci (Rome) 38:1094–1099, 1968

(25) PATHAK MA, KRÄMER DM: Photosensitization of skin in vivo by furocoumarins (psoralens). Biochim Biophys Acta 195:197–206, 1969

(26) COLE RS: Light-induced cross-linking of DNA in the presence of a furocoumarin (psoralen). Studies with a phage lambda, *Escherichia coli* and mouse leukemia cells. Biochim Biophys Acta 217:30–39, 1970

(27) DALL'ACQUA F, MARCIANI S, RODIGHIERO G: Interstrand cross-linkages occurring in the photoreactions between psoralen and DNA. FEBS Lett 9:121, 1970

(28) MORTZAWI SA: Meladinine mit UVA bei Vitiligo, Psoriasis, Parapsoriasis und Acne vulgaris. Derm Monatsschr 158: 908–909, 1972

(29) TRONNIER H, SCHULE D: First results of therapy with longwave UV after photosensitization of skin. Presented at the Sixth International Congress of Photobiology, Bochum, Federal Republic of Germany, 1972

(30) BORDIN F, BACCICHETTI F, MUSAJO L: Inhibition of nucleic acid synthesis in Ehrlich ascites tumor cells by irradiation in vitro in the presence of skin-photosensitizing furocoumarins. Experientia 28:148, 1972

(31) LEVIN RE, PARRISH JA: Phototherapy of vitiligo. Lighting Design Application 5:35–43, 1975

(32) GILCHREST BA, PARRISH JA, TANENBAUM L, et al. Oral methoxsalen photochemotherapy of mycosis fungoides. Cancer 38:683–689, 1976

(33) REED WB, SUGERMAN GE, MATHIS RA: DeSanctis-Cacchione syndrome. A case report with autopsy findings. Arch Dermatol 113:1561–1563, 1977

(34) DUBERTRET L, AVERBECK D, ZAJDELA F, et al: Photochemotherapy (PUVA) of psoriasis using 3-carbethoxypsoralen, a noncarcinogenic compound in mice. Br J Dermatol 101:379–389, 1978

(35) WOLFF K: PUVA 1979, Klinik und Praxis. *In* Fortschritte der Praktischen Dermatologie und Veneralogie (Braun-Falco O, Wolff HH, eds), vol 9. Berlin, Heidelberg: Springer-Verlag, 1979, pp 129–138

(36) MOSHER DB, PATHAK MA, FITZPATRICK TB: Vitiligo: Etiology, pathogenesis and treatment. *In* Update: Dermatology in General Medicine (Fitzpatrick TB, Eisen AZ, Wolff K, et al, eds). New York: McGraw-Hill, 1983, pp 205–225

(37) MORISON WL, PARRISH JA, FITZPATRICK TB: Oral methoxsalen photochemotherapy of recalcitrant dermatoses of the palms and soles. Br J Dermatol 99:293–302, 1978

(38) LEVINE MJ, PARRISH JA, FITZPATRICK TB: Oral methoxsalen photochemotherapy (PUVA) of dyshidrotic eczema. Acta Derm Venereol (Stockh) 61:570–571, 1981

(39) MORISON WL, WHITE HA, GONZALEZ E, et al: Oral methoxsalen photochemotherapy of uncommon photodermatoses. Acta Derm Venereol (Stockh) 59:366–368, 1979

(40) PARRISH JA, LEVINE MJ, MORISON WL, et al: Comparison of PUVA 8-methoxypsoralen plus UV light and beta carotene in the treatment of polymorphous light eruption. Br J Dermatol 100:187–192, 1979

(41) LOWE NJ, CRIPPS DJ, DUFTON PA, et al: Photochemotherapy for mycosis fungoides: A clinical and histological study. Arch Dermatol 115:50–53, 1979

(42) MORISON WL, PARRISH JA, FITZPATRICK TB: Oral psoralen photochemotherapy of atopic eczema. J Invest Dermatol 67:561, 1976

(43) HÖNIGSMANN H, GSCHNAIT F, KONRAD K, et al: Photochemotherapy for pustular psoriasis (von Zumbusch). Br J Dermatol 97:119–126, 1977

SESSION II

In Vitro and In Vivo Molecular Bases of Psoralen and Longwave Radiation Photosensitization Reactions

Photoreactive States of Furocoumarins[1, 2]

Pill-Soon Song[3]

ABSTRACT—On the basis of spectroscopic and wave-function analyses of the excited states of psoralens, it is predicted that the photocycloaddition at the 3,4-double bond leading to the formation of a monoadduct proceeds from both the singlet and triplet excited states, with the latter state being the preferential route, especially in the reactions between psoralens and nucleosides and nucleotides in solution. The photocycloaddition at the 4′,5′-double bond is likely to proceed by way of the singlet path exclusively. It is also predicted that the second photocycloaddition of the 3,4- or 4′,5′-monoadduct yielding cross-linked diadducts takes the singlet route. — Natl Cancer Inst Monogr 66: 15-19, 1984.

What determines the reactivity of the excited states of psoralens? The photoreactivity of psoralens with respect to the cycloaddition to DNA is determined by three main factors: kinetic, electronic, and steric. I describe these factors to elucidate the photoreactivity of psoralens in their excited states. However, the steric factor will be discussed only briefly.

KINETIC FACTORS

Lifetime of the Excited State

Figure 1 shows a Jablonski energy level diagram for psoralen. In the absence of bimolecular quenching and photocycloaddition, the lifetimes of the singlet and triplet states are given by the following expressions, respectively:

$$\tau_S = \frac{1}{k_F + k_{IC} + k_{ISC}},$$

$$\tau_T = \frac{1}{k'_{ISC} + k_P},$$

$$\simeq \frac{1}{k'_{ISC}} \quad \text{(at room temperature).}$$

The τ_S value ranges from 1 to 5 nanoseconds for psoralens [e.g., 1.8 nanoseconds for 8-MOP (*1, 2*)], whereas the τ_T value ranges from 1 microsecond to 1 second [e.g., 0.72 second for 8-MOP in ethanol at 77° K (*3, 4*)].

Unlike a reaction in the dark, photoreactions proceed with the excited states of limited lifetimes. Thus the longer the lifetime, the more kinetically reactive is the excited state because the steady-state concentration of the reacting state species is greater.

Coumarin serves as a model compound in a description of the excited states of psoralens, as will be illustrated later. Coumarin is only weakly fluorescent in polar solvents, and its τ_S is in the subnanosecond range (*3*). A short singlet lifetime makes the singlet-excited coumarin kinetically unreactive with respect to cycloaddition to Thy base in solution because the decay of the singlet state is much faster than the diffusion of the photoexcited coumarin toward substrate (Thy), which is a prerequisite for the eventual cycloaddition reaction. On the other hand, 5,7-DMC possesses a considerably higher fluorescence quantum yield ($\Phi_F \sim 0.65$) and longer lifetime ($\tau_S \sim 7.2$ nanoseconds) than does coumarin (*5*). Thus it is not surprising that 5,7-DMC photoreacts with a pyrimidine base through the singlet excited state (*6*).

Coumarin shows a high triplet yield by k_{ISC} from the singlet excited state (fig. 1). The coumarin triplet is long-lived and exhibits high reactivity for cycloaddition (particularly photodimerization). On the other hand, 5,7-DMC photodimerized preferentially from the singlet state (*6*).

Unlike coumarin, psoralens generally have longer τ_S values, as mentioned earlier. Thus it is possible that psoralens photodimerize and photocycloadd by way of the singlet excited state route. However, because of an efficient ISC, a psoralen is likely to undergo reactions with itself or Thy base in solution by the triplet state. When psoralens are intercalated in DNA, the singlet reaction can be a predominant path because the singlet-excited psoralen intercalated need not diffuse too far to encounter its substrate (Thy base).

Complexation and Intercalation

It was suggested above that the reactivity of an excited state is limited by the lifetime of that state because the excited state reaction entails a diffusional encounter with a substrate ($k_{\text{diffusion}} \simeq 10^9 \sim 10^{10}\ M^{-1}$ second^{-1} in solution at room temperature). The kinetic restriction imposed by a short lifetime of the excited state can be overcome if the diffusional prerequisite for the photocycloaddition of psoralen to Thy base is relaxed. This is true with the intercalated psoralen in DNA, when the psoralen is excited

ABBREVIATIONS: τ_S = fluorescence lifetime; 8-MOP = 8-methoxypsoralen; ISC = intersystem crossing; DMC = 5,7-dimethoxycoumarin.

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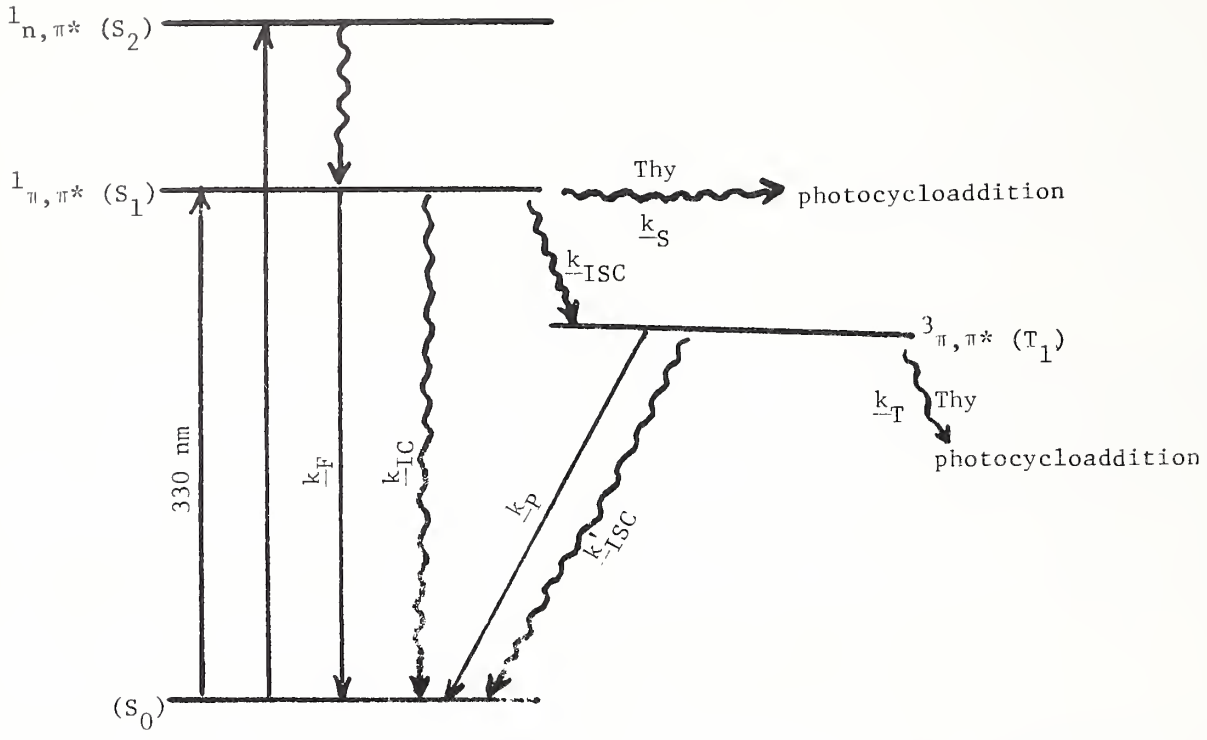

FIGURE 1.—Energy level diagram of the excitation of psoralen to the singlet excited S_1 and S_2 states and the relaxation from the excited states. k_F, rate constant for fluorescence; k_{IC}, internal conversion; k_{ISC}, intersystem crossing; k_S and k_T, cycloaddition in the singlet and triplet states, respectively.

in situ, and the excited species then reacts with Thy base in the intercalative proximity (*2*). This is equivalent to the reaction rate enhancement with the increase in substrate concentration, i.e., the *effective* Thy concentration in DNA–psoralen complexes is higher than the free Thy concentration in solution by several orders of magnitude.

In view of the above remarks, it is not surprising that, among all available coumarin derivatives studied, 5,7-DMC alone photocycloadds to DNA (monoadduct), as only this coumarin derivative intercalates to DNA (*7*). Apparently, 5,7-DMC meets steric requirements for intercalation analogous to 5-MOP, which also effectively intercalates to DNA (fig. 2).

Exciplex Formation

The probability of the photocycloaddition of psoralen to Thy in solution is enhanced if the excited singlet state of the former encounters the latter and forms an exciplex.

$$P_S + h\nu \rightarrow {}^1P_S^*,$$

$${}^1P_S^* + \text{Thy} \rightleftarrows {}^1P_S^* \cdot \text{Thy},$$

$${}^1P_S^* \cdot \text{Thy} \rightarrow \text{cycloadducts}.$$

An exciplex formation in DNA–psoralen complexes may also occur, thus effectively competing with the ISC (fig. 1) of the singlet psoralen. If this occurs, the photocycloaddition by the triplet route will be minimized. However, we have no experimental evidence for or against the exciplex effect in the photoaddition of psoralens to DNA.

ELECTRONIC FACTORS

Genealogy of the Absorption Spectra of Psoralens

The intrinsic photoreactivity of psoralens is determined by the electronic structure of the lowest excited states (S_1 and T_1). The electronic structure of the singlet excited states evolves from the electronic spectra of psoralens by the

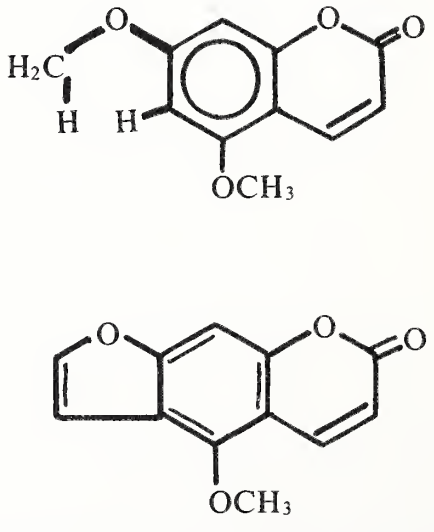

FIGURE 2.—Structural scheme of 5,7-DMC (*upper*) and 5-MOP (*lower*).

absorption of proper wavelengths of light. As mentioned earlier, coumarin serves as a useful model for the description of the electronic spectra of psoralens due to a close resemblance between the absorption spectra of psoralen and coumarin (*8*).

The UVA bands of coumarin and psoralen arise from their pyrone moiety (*7*). The absorption spectrum of psoralen can also be correlated with that of *o*-coumaric acid, which retains the pyrone moiety in an open form (fig. 3). *o*-Coumaric acid absorbs at 275 and 336 nm, similar to psoralen, but these 2 bands collapse into a single absorption maximum at 318 nm (*9*). The hydrogenation of the pyrone 3,4-double bond abolishes the long wavelength UV band of psoralens (spectrum not shown). However, a 3,4-dihydropsoralen retains the absorption corresponding to the second band of psoralens. On the other hand, the removal of the furyl moiety does not abolish the first absorption band of psoralens. In fact, upon opening of the pyrone ring, this band is still retained, as shown in the absorption spectrum of *o*-coumaric acid (fig. 3). Thus the UVA and UVB absorption bands of psoralens can be described either in pyrone moiety or by analogy of *o*-coumaric acid.

Effect of the (n,π^*) State on Photoreactivity

The carbonyl groups of coumarins and psoralens provide the sole source of the $^1(n,\pi^*)$ state, which is not readily resolved in the absorption spectra of these compounds. In coumarin, the $^1(n,\pi^*)$ state lies just above the lowest $^1(\pi,\pi^*)$ state, as one can deduce its approximate disposition from the broadness of the UVA absorption band and the extremely short lifetime of fluorescence. Direct evidence for the closeness of the $^1(n,\pi^*)$ and $^1(\pi,\pi^*)$ states in coumarin has been demonstrated. The fluorescence emission spectrum is structured, and its quantum yield and lifetime are greatly enhanced as a result of the coordination of the positive lithium ion with the coumaryl carbonyl group (*10*). Hence the absorption band in the UVA region also becomes structured. These results are due to a weakening of the vibronic interaction between the $^1(n,\pi^*)$ and $^1(\pi,\pi^*)$ states, as the $^1(n,\pi^*)$ state is raised in energy by the lithium coordination. The 5,7-methoxy groups exert a similar hypsochromic effect on the $^1(n,\pi^*)$ state of coumarin, resulting in an overall lengthening of the $^1(\pi,\pi,^*)$ state. The consequence of the separation between the $^1(n,\pi^*)$ and $^1(\pi,\pi^*)$ states is that the excited (π,π^*) state of 5,7-DMC is kinetically (i.e., long τ_S) and electronically (i.e., purer π,π^* character for cycloaddition at the 3,4-bond) more reactive than the parent coumarin.

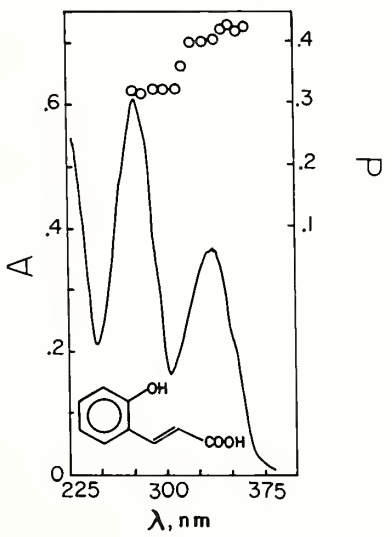

FIGURE 3.—The absorption (A, absorbance) spectrum of *o*-coumaric acid in ethanol at 77° K. We measured fluorescence excitation polarization (P, *open circles*) to distinguish the first and second absorption bands according to their relative polarization directions. Because the P values of the 2 bands differ by less than 0.15, as in coumarins and psoralens, the polarization directions are not too far from being mutually parallel. λ = wavelength.

The relatively long lifetime (τ_S) of the $^1(\pi,\pi^*)$ state in psoralens (fig. 1) can be explained in the same manner. In particular, electron-donating substituents, such as methyl (as in trimethylpsoralen) and methoxy (as in 8-MOP) groups, will further raise the $^1(n,\pi^*)$ state and thus enhance the photocycloaddition by way of the singlet state. Electron-withdrawing groups tend to lower the $^1(n,\pi^*)$ state, and, therefore, the photoreactivity of the $^1(\pi,\pi^*)$ state of 3-carbethoxypsoralen may be significantly lower than that of the $^3(\pi,\pi^*)$ state in this derivative and the $^1(\pi,\pi^*)$ state in other psoralens.

Another consequence of close-lying $^1(n,\pi^*)$ state is the enhancement of ISC to the $^3(\pi,\pi^*)$ state through vibronic spin-orbit coupling and through the intervening $^3(n,\pi^*)$ state, which is positioned between the $^1(\pi,\pi^*)$ and the $^3(\pi,\pi^*)$ states (*3*). Therefore, it is possible that the $^3(\pi,\pi^*)$ state of 3-carbethoxypsoralen exhibits higher reactivity than does its $^1(\pi,\pi^*)$ state, at least kinetically, i.e., high triplet yield. However, the $^3(\pi,\pi^*)$ state of 3-carbethoxypsoralen is not necessarily reactive in its electronic reactivity indices.

Wave-function Analysis of the Excited States

At least 2 electronic factors are likely to determine the photocycloadditive reactivity of 3,4- and 4′,5′-double bonds of psoralens (i.e., $C_3{=}C_4$ and $C_{4'}{=}C_{5'}$, respectively; fig. 4). These factors are the degree of activation (local excitation) and the electron density of the 3,4- and 4′,5′-groups. The former is achieved by the excitation of the reactive region of the psoralen π-electron network by the absorption of light, whereas the electron density of the reactive region (3,4- and 4′,5′-) is enhanced by an intramolecular charge transfer to the reactive double bond (from the rest of the π-electron moiety). Thus the photoreactivity of 5,7-DMC is attributable to a strong charge transfer from the substituent groups to the $C_3{=}C_4$ bond, making the latter group electron rich. These qualitative descriptions can be put forth in a quantitative way if one analyzes the wave functions of the excited states of psoralens, using the method of configuration analysis (*11, 12*).

Configuration analysis of the $^1(\pi,\pi^*)$ state of coumarin and psoralen suggests that this state possesses significant charge transfer character in the $C_3{=}C_4$ bond of the pyrone

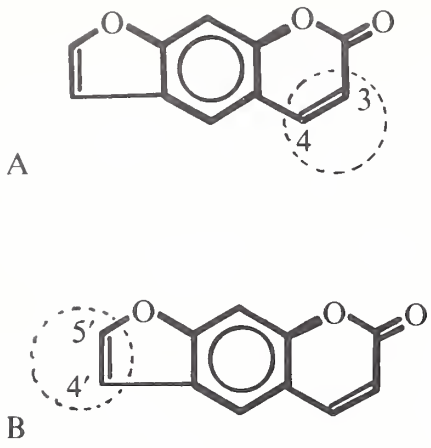

FIGURE 4.—Fragmentation patterns for the wave-function analysis of psoralen by the configuration analysis method. A) Psoralen ≡ benzofuryl formate + ethylene. B) Psoralen ≡ 7-hydroxycoumaryl + ethylene.

ring; 32% of the charge transfer is from the phenylformate moiety to the pyrone C=C vacant molecular orbital in coumarin and 22% in psoralen. Thus the singlet excited state becomes electron rich at the C=C bond, relative to the ground state, as the C=C bond becomes photochemically reactive (fig. 4).

The C_3=C_4 bond of coumarin is locally excitable [7.2% of the $^1(\pi,\pi^*)$ state wave function is localized at this bond], whereas virtually no local excitation is predicted in psoralen. The singlet reactivity of the C_3=C_4 bond is attributable to the charge transfer effect mentioned above.

Configuration analysis of psoralen also shows that the $C_{4'}$=$C_{5'}$ bond of the furyl moiety becomes only slightly electron rich due to the charge transfer from the 7-hydroxycoumaryl moiety to the ethylenic unit (fig. 4); the charge transfer contribution amounts to 4%. The reactivity of the $C_{4'}$=$C_{5'}$ bond is determined by kinetic and steric factors rather than by electronic factors. From the calculations described above, one can conclude that the C_3=C_4 bond is generally more reactive than is the $C_{4'}$=$C_{5'}$ bond.

The triplet $^3(\pi,\pi^*)$ state is highly localized at the C_3=C_4 bond [as much as 41% of the wave function is localized; (*12*)]. In addition, this bond becomes preferentially rich in electron density due to the charge transfer effect, whereas the $C_{4'}$=$C_{5'}$ bond is neither locally excited nor electron rich.

From these results, it appears that triplet photoreactivity resides with the pyrone rather than with the furyl moiety. In fact, configuration analysis shows that the triplet $^3(\pi,\pi^*)$ state of psoralen may be adequately described by 7-hydroxycoumaryl moiety (*12*).

CONCLUSIONS

The photoreactivity of psoralens is governed by many factors. On the basis of the analyses given here, one can predict that the singlet excited state, $^1(\pi,\pi^*)$, will be generally reactive with respect to photocycloaddition, particularly if the psoralen molecule intercalated in the DNA is photoexcited. However, there is a good chance that the triplet state is reactive with respect to the monoaddition of psoralens at the C_3=C_4 bond, particularly in a free solution of psoralens and substrates.

Monoaddition at C_3=C_4 results in a low-lying triplet state (*7*); this state (π,π^*) is probably unreactive with respect to the second photocycloaddition (cross-link formation) because of its rapid relaxation to the ground state. On the other hand, monoaddition at $C_{4'}$=$C_{5'}$ yields a relatively long-lived $^1(\pi,\pi^*)$ state. Therefore, the cross-link formation after monoaddition at C_3=C_4 or $C_{4'}$=$C_{5'}$ is likely to proceed from the singlet excited state.

REFERENCES

(*1*) POPPE W, GROSSWEINER LI: Photodynamic sensitization by 8-methoxypsoralen via the singlet oxygen mechanism. Photochem Photobiol 22:217–219, 1975

(*2*) SONG P-S, TAPLEY KJ JR: The photochemistry and photobiology of psoralens. Photochem Photobiol 29:1177–1197, 1979

(*3*) MANTULIN WW, SONG P-S: Excited states of skin-sensitizing coumarins and psoralens: Spectroscopic studies. J Am Chem Soc 95:5122–5129, 1973

(*4*) BENSASSON RV, LAND EJ, SALET C: Triplet excited state of furocoumarins: Reaction with nucleic acid bases and amino acids. Photochem Photobiol 27:273–280, 1978

(*5*) SHIM SC, CHOI KY, SONG P-S: Studies on the phototoxicity of coumarin derivatives. 1. Photocyclodimerization of 5,7-dimethoxycoumarin. Photochem Photobiol 27:25–31, 1978

(*6*) SHIM SC, CHAE KH: Photocycloaddition of 5,7-dimethoxycoumarin to thymine. Photochem Photobiol 30:349–353, 1979

(*7*) OU CN, TSAI CH, SONG P-S: Excited states of skin-sensitizing psoralens and their reactions with nucleic acids. *In* Research in Photobiology (Castellani A, ed). New York: Plenum Press, 1977, pp 257–265

(*8*) MOORE TA, HARTER ML, SONG P-S: Ultraviolet spectra of coumarins and psoralens. J Mol Spectrosc 40:114–157, 1971

(*9*) SONG P-S, SHIM SC, MANTULIN WW: The electronic spectra of psoralens in their ground and triplet excited states. Bull Chem Soc Jpn 54:315–316, 1981

(*10*) SONG P-S, CHAE Q: Anomalous effects of heavy atom salts and temperature dependence of fluorescence in retinylic and tetrapyrrole (phytochrome) polyenes. J Luminesc 12/13:831–837, 1976

(*11*) SONG P-S, CHIN CA, YAMAZAKI I, et al: Electronic structure and photoreactivity of the triplet states of skin-sensitizing psoralens. Int J Quantum Chem, Quantum Biol Symp No 2:1–8, 1975

(*12*) CHIN CA: Theoretical analyses of electronic excited states of chlorophylls, carotenoids, firefly luciferins, and psoralens. Ph.D. dissertation, Texas Tech Univ, Lubbock, Texas, 1975

DISCUSSION

L. Grossweiner: Dr. Song, was the first reactive state of psoralens the excited singlet or the triplet state?

P-S. Song: What I said was that, with the psoralen, the 4′,5′-addition would be most likely by way of the singlet state, and for the 3,4-addition, there may be a higher probability of triplet addition. However, that does not

exclude singlet addition, although it does exclude singlet addition in coumarin. That is what I was trying to say. If you modify coumarin by dimethoxy substitution, then the singlet addition becomes predominant.

Grossweiner: There is photochemical evidence that triplet states of 8-MOP and trimethylpsoralen do not react with DNA or with pyrimidines. That is by laser flash photolysis work, although psoralen triplet states do. Some investigators doing laser flash photolysis work are looking at triplet reactivity directly and find that 8-MOP and trimethylpsoralen triplet states are nonreactive.

Song: Are you referring to the quenching of the triplet state?

Grossweiner: Yes.

Song: This does not necessarily represent an addition reaction.

Grossweiner: Does psoralen quenching?

Song: I believe you are referring to data by Land et al.

Grossweiner: That is right.

Song: I think those data do not represent the photochemical reactions. Well, perhaps a tiny fraction.

Grossweiner: Yes. Mostly physical quenching.

In Vitro Characterization of the Reaction of Four Psoralen Derivatives With DNA[1, 2]

Stephen T. Isaacs,[3, 4] Gary Wiesehahn,[3, 5] *and* Lesley M. Hallick[3, 6, 7]

ABSTRACT—Four psoralen derivatives were radiolabeled and used for in vitro DNA binding studies. The derivatives were compared for their dark-binding ability to DNA, photoreactivity, and for unwinding angles. The dark-binding dissociation constants we determined were 1.4×10^{-3} *M* for 8-methoxypsoralen (8-MOP), 3.5×10^{-4} *M* for 5-methoxypsoralen (5-MOP), and 5.5×10^{-4} *M* for 5-methylisopsoralen (5-MIP). We did not detect any dark binding to DNA for 3-carbethoxypsoralen (3-CP). Photoaddition experiments indicated that the relative rates of photoaddition by psoralen to DNA (measured as psoralens bound per base pair per second) are 4.4×10^{-5} for 5-MIP, 9.2×10^{-6} for 5-MOP, 7.8×10^{-6} for 8-MOP, and 4.6×10^{-6} for 3-CP. We found the peak level of binding (for an initial base pair-to-psoralen ratio of 22) to be 27, 32.2, 31.2, and 1,538 base pairs per psoralen bound for 5-MOP, 8-MOP, 5-MIP, and 3-CP, respectively. In addition, 3-CP adducts could be photoreversed by prolonged irradiation at 360 nm. After 10 hours of irradiation, the amounts of 3-CP bound to DNA had fallen to less than 50% of the peak amount bound. In the same time, the amount of 8-MOP and 5-MOP bound had fallen to 95% of their peak values, and 5-MIP had fallen to 85% of its peak value. We also performed unwinding angle experiments to determine the amount of unwinding of the DNA helix induced per photobound derivative molecule; the unwinding angles ± 3° were 25 for 5-MOP, 28 for 8-MOP, 26 for 3-CP, and 18 for 5-MIP. — Natl Cancer Inst Monogr 66: 21–30, 1984.

Psoralens are bifunctional nucleic acid photoreagents which upon irradiation with long-wavelength UV light form both monoadducts and diadducts with pyrimidine bases in DNA and RNA (*1–3*). The reaction sequence for diadduct formation involves 3 discrete steps: 1) intercalation of the psoralen between the base pairs in double-stranded regions of the nucleic acid structure; 2) absorption of 320–380 nm light by the psoralen resulting in covalent photocycloaddition of the 4′,5′- or the 3,4-double bond to the 5,6-double bond of the pyrimidine, forming a monoadduct; and 3) a second photocycloaddition of the monoadduct to a second pyrimidine situated on the opposite strand resulting in a diadduct. With psoralen, 8-MOP and 4,5′,8-TMP, only the 4′,5′-monoadduct is competent to form a diadduct with 320–380 nm light; the 3,4-monoadduct does not absorb in this region, and, consequently, cannot form a diadduct upon further irradiation.

We initiated the present study to establish the relative reactivity of 8-MOP, 5-MOP, 3-CP, and 5-MIP with DNA. The structures of the compounds are shown in figure 1. The parameters measured for each of the compounds were the dark-binding dissociation constant K_D, the rate constant k_2 for photoaddition to DNA, the peak level of adduct formed, and the unwinding angle of supercoiled DNA induced by each of the psoralens. The measured unwinding angle yielded information about the nature of the noncovalent complex.

Historically, psoralens have been used with UV light for the treatment of various skin diseases including vitiligo (*4*), leukoderma (*4*), mycosis fungoides (*5*), and more recently, for the photochemotherapy of psoriasis (*6*). The relationship between the sanative effect of psoralens and their nucleic acid binding properties remains unclear, as does their potential for acting as mutagenic and carcinogenic agents. The psoralen derivatives evaluated in this study for in vitro reactivity with DNA were studied concurrently in vivo by other investigators to establish their oncogenic potential with and without light.[2] The complementary results of these 2 studies will allow the clinical use of these compounds to be viewed at the molecular and medicinal levels.

MATERIALS AND METHODS

Synthesis of the radiolabeled psoralens.—The proton magnetic resonance spectra were obtained at room temperature in deuterated chloroform with tetramethylsilane as an internal standard on a T60 NMR Spectrometer from Varian Associates (Palo Alto, Calif.); chemical shifts were expressed in parts per million. Mass spectra were obtained with an AEI MS-12 Mass Spectrometer purchased from AEI Scientific Apparatus, Inc. (Elmsford, N.Y.); ion potential was 70 volts; probe temperature was 120–170° C.

ABBREVIATIONS: 8-MOP = 8-methoxypsoralen; 5-MOP = 5-methoxypsoralen; 4,5′,8-TMP = 4,5′,8-trimethylpsoralen; 3-CP = 3-carbethoxypsoralen; 5-MIP = 5-methylisopsoralen; TLC = thin-layer chromatography; HPLC = high-performance liquid chromatography: sp act = specific activity; W = watt(s); Col E1 = colicin E1; HMT = 4′-hydroxymethyl-4,5′,8-trimethylpsoralen.

[1] Presented at the Conference on Photobiologic, Toxicologic, and Pharmacologic Aspects of Psoralens held in Research Triangle Park, North Carolina, March 1–3, 1982.

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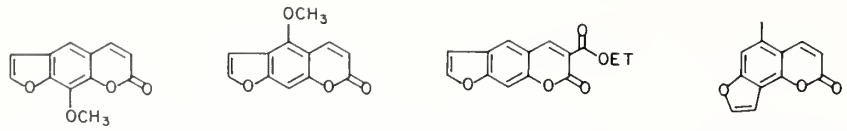
[7] We thank Drs. John E. Hearst and Henry Rapoport for their valuable scientific contributions to this study and gratefully acknowledge the technical assistance of Ms. Kathryn Macbride.



8-Methoxypsorelen 5-Methoxypsorelen 3-Carbethoxypsorelen 5-Methylisopsorelen

FIGURE 1.—Structures of the psoralen derivatives.

Radioactivity measurements were made in an LS-230 liquid scintillation counter obtained from Beckman Instruments Inc. (Mountain View, Calif.). We (*7*) determined specific activities by counting aliquots of standard solutions of each tritiated psoralen in Omnifluor-Triton X-100-toluene containing 10% water (vol/vol). The concentration of the standard solutions was determined with extinction coefficients determined in this laboratory. These coefficients are 2.4×10^4 (5-MIP, 249 nm); 2.6×10^4 (3-CP, 249 nm); 1.8×10^4 (5-MOP, 249 nm); and 2.3×10^4 (8-MOP, 247 nm). Radiochemical purity was determined by a combination of TLC and HPLC. We ran the TLC of each purified product in at least 3 solvent systems (chloroform; chloroform/methanol, 98.2; benzene/acetone, 1:1) using No. 13181 silica gel plates from Eastman Chemicals, Inc. (Rochester, N.Y.) with fluorescent indicator. Each plate was divided into equal sections which were placed into scintillation vials, and the amount of radioactivity in each section was determined. Radiochemical purity is expressed as the percent of the total radioactivity found in the section which contained the tritiated compounds. The HPLC of the labeled psoralens was performed with the use of an Altex/Beckman 320MP liquid chromatograph, equipped with a Beckman Model 105 variable wavelength detector, both from Beckman Instruments, Inc. (Berkeley, Calif.). Reverse-phase octadecylsilane columns (10×250 mm or 4.6×250 mm 5 μ ultrasphere) were used with a water–methanol gradient for elution. Fractions were collected from the gradient, counted, and the radiochemical purity determined from the percent of the total radioactivity found coincident with the peak corresponding to the tritiated psoralen. We purchased the tritiated methyl iodide (2.0–2.5 Ci/mmol) from New England Nuclear (Boston, Mass.) and the boron tribromide from Alfa Inorganics (Danvers, Mass.); carrier-free tritium gas was provided by the Lawrence Berkeley Laboratory (Berkeley, Calif.). Chromatography columns were run with 60- to 200-mesh Baker silica gel. The TLC plates were visualized with 254-nm light. All solvents and chemicals used were of reagent grade or better. Unlabeled 8-MOP and 3-CP, provided by the National Cancer Institute, were analyzed and no major impurities were found; these compounds were used as received. Unlabeled 5-MOP (also from the Institute) contained a contaminant, tentatively identified as 5,8-dimethoxypsoralen, which was removed chromatographically before use (silica gel column, elution with methylene chloride). Unlabeled 5-MIP was synthesized from 5-methylresorcinol by a new procedure that will be reported elsewhere.

8-([$^3H_3$]-Methoxy)psoralen.—The 8-MOP, with a radiochemical purity of 98.7% and a sp act of 2.5 Ci/mmol, was synthesized as described in (*7*).

5-([$^3H_3$]-Methoxy)psoralen.—Unlabeled 5-MOP was demethylated with boron tribromide as described for 8-MOP (*7*) providing 5-hydroxypsoralen (93%): mass spectrum m/z (relative intensity) 202 (M+, 100). The calculated values for 5-hydroxypsoralen ($C_{11}H_6O_4$) are C, 65.4; H, 3.0. Analyzed values obtained were C, 65.2; H, 3.1. The 5-hydroxypsoralen (20.0 mg, 0.099 mmol); anhydrous potassium carbonate (40.0 mg, 0.29 mmol); tritiated methyl iodide (4.1 mg, 0.028 mmol); and dry acetone (2 ml, dried over 4A sieves) were placed in a low-pressure bomb apparatus (*7*) and heated at 55° C for 38 hours while it was magnetically stirred. After this period, the solvent was removed by evaporation under vacuum, followed by the addition of 5 ml each of chloroform and water. The chloroform was separated and the aqueous phase re-extracted three times with 3 ml chloroform. Extracts were combined, washed twice with 5 ml water, and then run through 2 Pasteur pipettes filled with anhydrous sodium sulfate. By TLC (chloroform, methylene chloride) of the dry extract, we found one spot which co-chromatographed with authentic 5-MOP. The solvent was evaporated, and the residual white solid dissolved in 1 ml chloroform, it was then loaded on a 0.5×8-inch silica gel column and eluted with methylene chloride. The fractions containing the product were combined, the solvent was removed, and the product dissolved in 5 ml absolute ethanol. By radiochemical analysis, we detected the product to be greater than 98% radiochemically pure with a sp act of 2.1 Ci/mmol. The yield was essentially quantitative.

[4′,5′-$^3H_2$]-3-Carbethoxypsoralen.—Unlabeled 3-CP (63 mg, 0.24 mmol), 10% palladium on charcoal (37 mg), and glacial acetic acid (2 ml) were placed in a small round bottom flask, attached to a vacuum line, frozen with liquid nitrogen, and then the reaction vessel was evacuated. Carrier-free tritium gas was added to slightly below 1 atmosphere, and the round bottom flask was warmed briefly in a 60° C water bath to redissolve the 3-CP. The heterogeneous mixture was stirred at room temperature for 1 hour after which approximately 0.24 mmol tritium gas had been consumed. The mixture was frozen, the tritium gas was evacuated, 10 ml methanol was added, and the slurry was centrifuged for removal of the catalyst, after which the supernatant was decanted, frozen, and then lyophilized. The TLC (chloroform) of the residual solid revealed no starting material and a low R_F blue fluorescent spot that co-chromatographed with authentic 4′,5′-dihydro-3-CP. Crude [4′,5′-$^3H_2$]-4′,5′-dihydro-3-CP was dissolved in 0.5 ml chloroform/methanol 98:2, loaded on a 0.5×8-inch silica column, and eluted with chloroform/methanol 98:2. Fractions containing the product were combined, the solvent was removed, and the residue was first dissolved in diphenylether (5.0 ml) containing 10% palladium on charcoal (32 mg) and then refluxed for 17 hours. After this

period, TLC (methylene chloride) showed that most of the starting material had been converted to [4′,5′-$^3H_2$]-3-CP as determined by co-chromatography with authentic 3-CP. The product was isolated from the reaction mixture by column chromatography (methylene chloride), diluted with unlabeled 3-CP (5.4 mg), and a final column was run (methylene chloride). Product fractions were combined, and the solvent was evaporated under vacuum. The residual solid was dissolved in absolute ethanol (10 ml) for radiochemical analysis, which demonstrated the product to be 98.5% radiochemically pure with a sp act of 3.0 Ci/mmol; the yield was approximately 5% for the 2-step procedure.

In a parallel experiment, 3-CP was reduced with hydrogen gas to 4′,5′-dihydro-3-CP and then dehydrogenated as above back to 3-CP. The proton magnetic resonance, UV, and mass spectra of the 3-CP prepared in this manner were identical to the authentic compound.

[4′,5′-$^3H_2$]-5-Methylisopsoralen.—Unlabeled 5-MIP (58 mg, 0.29 mmol), 10% palladium on charcoal (29 mg), and glacial acetic acid (7.0 ml) were placed in a small round bottom flask and reduced with tritium gas in the same way as for 3-CP. Approximately 0.31 mmol of tritium gas was consumed in the reaction. Following lyophilization, TLC (chloroform) of the residue revealed unreacted starting material, a low R_F blue fluorescent spot corresponding to [4′,5′-$^3H_2$]-4′,5′-dihydro-5-MIP and a high R_F nonfluorescent spot corresponding to [3,4,4′,5′-$^3H_4$]-3,4,4′,5′-tetrahydro-5-MIP. The dihydro compound was isolated by column chromatography on a 0.5 × 8-inch silica column (methylene chloride). After this purification, [4′,5′-$^3H_2$]-4′,5′-dihydro-5-MIP (30 to 40 mg, 0.2 mmol); 10% palladium on charcoal (32 mg); and diphenylether (5.0 ml) were placed in a small round bottom flask and refluxed for 28 hours. Results of TLC (methylene chloride) indicated that most of the starting material had been converted to [4′,5′-$^3H_2$]-5-MIP as determined by co-chromatography with authentic 5-MIP. The product was purified by chromatography on 2 silica columns (methylene chloride). The fractions containing the purified product were combined, the solvent was evaporated, and the residue dissolved in absolute ethanol; this product was 99% radiochemically pure with a sp act of 7.4 Ci/mmol. A summary of the radiochemical data pertaining to the tritium-labeled psoralens is given in table 1.

Determination of dark-binding dissociation constants.—Equilibrium dialysis was performed as described by Hyde and Hearst (*8*). Sonicated calf thymus DNA obtained from Sigma Chemical Co. (St. Louis, Mo.) was used at a concentration of 25 μg/ml, and all derivatives were used at a psoralen-to-DNA ratio of 1:30 base pairs. All dialysis experiments were performed in 10 m*M* Tris–HCl, 1 m*M* EDTA, pH 8.0. The initial concentration of unlabeled psoralen was adjusted to equivalent concentrations inside and outside the dialysis bag. A trace amount of labeled psoralen was then added to either the inside or outside of the bag. Aliquots of the outside solution were counted daily until the counts on the outside of the duplicate samples were equivalent. The time needed for equilibration was typically 72 hours.

TABLE 1.—*Specific activity, radiochemical purity, and method of preparation of the [^3H]psoralen derivatives*[a]

Compound	Specific activity Ci/mmol	Radiochemical purity, %	Method of preparation
8-MOP	2.5	98.7	Demethylation–alkylation
5-MOP	2.1	98.0	Demethylation–alkylation
3-CP	3.0	98.5	Reduction–dehydrogenation
5-MIP	7.4	99.0	Reduction–dehydrogenation

[a] *See* figure 1 for structure and label position of respective psoralen derivatives.

Determination of photoreactivity of the derivatives with DNA.—Compounds were irradiated with a light source consisting of two 48-inch, 40 W Sylvania #FR 40/PUVA lamps with internal reflectors obtained from GTE-Sylvania (Stamford, Conn.). The lamps were mounted vertically 2 inches on either side of a quartz cuvette which contained the sample. The initial lamp intensities were 4.7 mW/cm^2 [measured with a Blak-Ray J-221 meter, Ultraviolet Products (San Gabriel, Calif.)]. The intensity slowly decreased to 3.5 mW/cm^2 after 150 hours of use. The cuvette temperature was maintained at 20° C by air cooling. Sonicated calf thymus DNA was present at a concentration of 250 μg/ml, and the psoralen derivatives were present at a drug-to-DNA ratio of 1:22 base pairs. All samples were irradiated in a buffer of 10 m*M* Tris–HCl, 1 m*M* EDTA, pH 8.0. Unbound drug was removed as described previously (*9*).

Determination of unwinding angles.—Supercoiled Col E1 DNA was isolated from *Escherichia coli* JC411 thy (Col E1) after chloramphenicol amplification of the plasmid (*10*), according to the procedure of Modrich and Zabel (*11*). The molecular weight of Col E1 DNA was taken to be 4.2×10^6 (*12*).

Supercoiled Col E1 DNA was totally relaxed in the presence of ethidium bromide (*9*) with the use of HeLa nicking–closing enzyme (a gift from J. Ruppell). A typical reaction mixture contained 176 μg of supercoiled Col E1 DNA, 1.41 μg of ethidium bromide, and 100 μl of nicking–closing enzyme in 2 ml of 0.125 *M* NaCl, 10 mM Tris–HCl, 2 m*M* EDTA, pH 8.0. After allowing the reaction to proceed for 24 hours at 20° C, we added sodium dodecyl sulfate to a final concentration of 1% and NaCl to a final concentration of 0.5 *M*. We extracted the DNA solution three times with Sevag (chloroform/isoamyl alcohol, 24:1) to remove ethidium and protein, and then precipitated the DNA with ethanol. Removal of the ethidium introduces superhelical turns in the DNA, making a population of partially relaxed, covalently closed circular DNA. (Our purpose in producing this partially relaxed DNA population was to move the superhelical DNA into a region of the agarose gel that is most sensitive to DNA superhelical density, so that the small changes induced by adduct formation could be detected.) Partially relaxed Col E1 DNA was photoreacted with each of the derivatives in

10 m*M* Tris–HCl, 1 m*M* EDTA, pH 8.0 with 1 hour of irradiation (15 min for 3-CP) in the light source described above. Each of the photoreacted samples was made 0.5 *M* in NaCl, extracted three times with Sevag, and precipitated twice with ethanol. The initial ratios of 3-CP to DNA needed to produce enough photoadducts for detectable DNA unwinding was 0.21 to 1.2 3-CP/base pair. Determinations of the number of photoadducts per Col E1 DNA molecule and agarose gel electrophoresis were performed as described in (*9*).

RESULTS

A tentative mechanism for the reaction of the psoralen derivatives with DNA is the following (*13*):

$$B \underset{k_{-3}}{\overset{h\nu\ k_3}{\rightleftharpoons}} P + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PS \underset{k_{-2}}{\overset{h\nu\ k_2}{\rightleftharpoons}} A.$$

In this model, P is the psoralen derivative in question, S is a psoralen binding site in the DNA, PS is the noncovalent intercalation complex between psoralen and the DNA, A refers to covalent adduct of the psoralen to the DNA, and B refers to photobreakdown products of the psoralen.

The parameters measured in this study were the noncovalent binding constant K_D, the photoaddition rate constant k_2, the peak level of covalent adduct A, and the degree of unwinding of double-stranded DNA induced by the photoaddition of each psoralen derivative. The unwinding measurement yields information on the nature of the noncovalent complex. The types of adduct formed, the rate of psoralen photobreakdown, and the nature of the photobreakdown products were not addressed.

Noncovalent Binding Dissociation Constants

The dissociation constant for the noncovalent binding of each psoralen derivative to DNA is defined by the expression:

$$K_D = \frac{k_{-1}}{k_1} = \frac{[P][S]}{[PS]},$$

where $[P]$ is the concentration of free psoralen, $[S]$ is the concentration of unoccupied binding sites where each base pair is taken to be a binding site, and $[PS]$ is the concentration of bound sites.

By determining the dissociation constant of each psoralen compound at a constant ratio of 1 psoralen for every 30 base pairs, we can measure the amount of each derivative associated with the DNA before irradiation. The extent of the noncovalent binding that occurs is a function of the structure of each of the compounds, and it affects the ultimate level of psoralen photobound to the DNA. At this psoralen-to-base pair ratio (1:30), the equilibrium measurement is made far enough from binding site saturation so that site exclusion is not a factor.

The noncovalent binding constants for 8-MOP, 5-MOP, and 5-MIP are presented in table 2. The percent dark bound refers to the percentage of psoralen derivative which is noncovalently associated with the DNA before irradiation. As shown in table 2, 5-MOP is the strongest binder, with approximately 10% of the compound dark bound to the DNA. Weaker binding occurred with 5-MIP (6.4%) and 8-MOP (2.5%), whereas no binding with 3-CP was detected. The sensitivity of the experimental method was such that a dissociation constant greater than 5×10^{-3} would not have been detected. This dissociation constant would correspond to a binding level of approximately 0.7%.

Photoaddition of the Psoralens to DNA

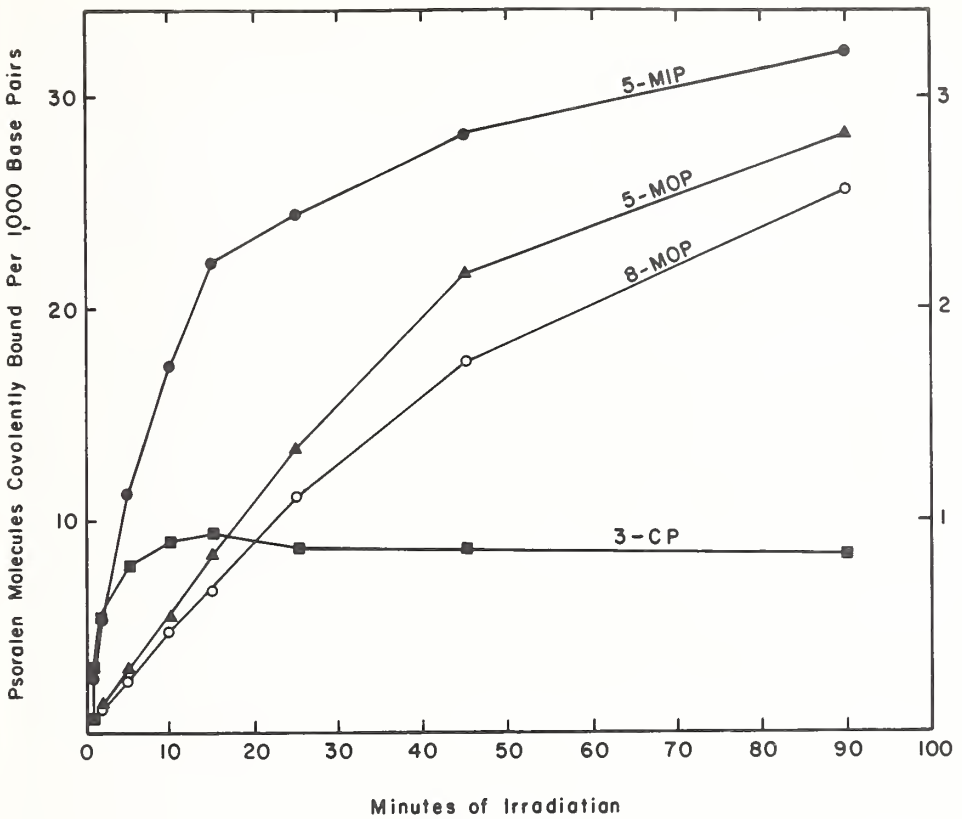
A time course of covalent photoaddition of each derivative to DNA is shown in figure 2; covalent binding data are summarized in table 2. All photoaddition experiments were performed at a ratio of 1 psoralen for every 22 DNA base pairs, a ratio similar to that used for the dark-binding experiments.

To investigate the kinetics of the photoaddition reaction, we irradiated DNA solutions containing each of the derivatives for various times and determined the amount of covalently bound derivative. In the initial stage of the reaction, covalent adduct formation is linear with time as shown in figure 2. The photoaddition rate constants k_2, determined graphically for each of the derivatives, indicated the highest rate of photochemical addition was exhibited by 5-MIP with a rate constant of 4.4×10^{-5} molecules·base pair·second. Both 5-MOP and 8-MOP reacted more slowly than did 5-MIP by factors of approximately 5 ($k_2 = 9.2 \times 10^{-6}$) and 6 ($k_2 = 7.8 \times 10^{-6}$), respectively. The slowest addition occurred with 3-CP ($k_2 = 4.6 \times 10^{-6}$), which reacted ten times more slowly than did 5-MIP.

TABLE 2.—*Noncovalent binding constants, photoaddition rate constants, and photoaddition plateau values for the psoralen derivatives*

Compound	K_D DNA, mol/liter	Percentage dark bound[a]	k_2[b]	Photoaddition plateau[c]	Percentage covalently bound[d]
8-MOP	1.4×10^{-3}	2.5	7.8×10^{-6}	31	68
5-MOP	3.5×10^{-4}	9.8	9.2×10^{-6}	37	81
3-CP	ND[e]	ND	4.6×10^{-6}	1	2
5-MIP	5.5×10^{-4}	6.4	4.4×10^{-5}	32	70

[a] Percentages are for added psoralen derivative dark (noncovalently) bound.
[b] Values are expressed as psoralens per 1,000 base pairs per second.
[c] Peak values of psoralen derivative bound to the DNA are in units of psoralen molecules per 1,000 base pairs.
[d] Percentages are of added psoralen derivative covalently bound.
[e] *ND* = none detected.



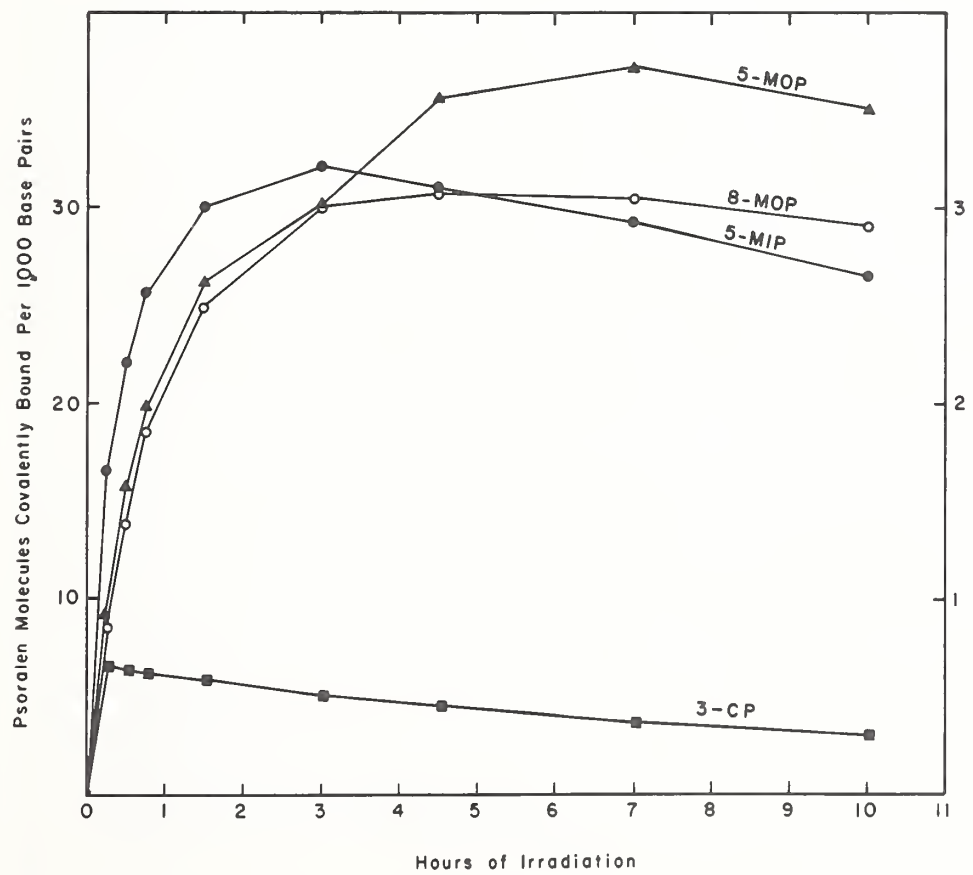


FIGURE 2.—Photochemical addition of the psoralen derivatives to DNA. *Upper* portion shows initial stage of the reaction where covalent adduct formation is linear with time, whereas the *lower* shows the peak binding level achieved by each derivative. *Left-hand ordinate* pertains to 8-MOP (○), 5-MOP (▲), and 5-MIP (●); *right hand ordinate* pertains to 3-CP (■).

To determine the maximum amount of each psoralen which can be photobound to the DNA, we continued the irradiation until the peak binding level for each derivative was achieved. At this point, further covalent addition cannot occur due to the depletion of the free psoralen pool by the addition and breakdown pathways. As shown in figure 2, the peak binding levels determined were in the order (psoralen/1,000 base pairs, percentage of psoralen bound): 5-MOP (37, 81); 5-MIP (32, 70); 8-MOP (31, 68); and 3-CP (1, 2). With 5-MOP, 5-MIP, and 8-MOP, the peak binding level exceeded the percent dark bound by several fold. This "pumping" effect results from replenishment of dark-bound complex with free psoralen from the solution as the photochemical addition proceeds. This implies that the rate of photoaddition to the DNA is faster than the rate of photobreakdown of the free compounds in solution. Such a correlation has been established for derivatives of 8-MOP and 4,5',8-TMP in a related study (*13*). Very little photoaddition of 3-CP was detected; the maximum amount bound was 0.6 to 0.9 3-CP/1,000 base pairs. This result agrees with evidence in the present study that no dark binding of 3-CP to DNA could be detected and that 3-CP has been reported to undergo rapid photodecomposition with 365-nm irradiation (*14*). Under these conditions, most of the 3-CP would be depleted through the photobreakdown path with little resultant photoaddition of 3-CP to DNA.

Each of the psoralen derivatives reached their peak bound levels at a characteristic time and, upon further irradiation, underwent photoreversion to varying degrees. Both 5-MOP and 8-MOP dropped approximately 5% from their peak bound values, whereas 5-MIP dropped about 15% after 10 hours of irradiation. The decrease for 3-CP was much larger, with approximately 50% of the initially bound compound removed from the DNA after 10 hours (fig. 2). Further irradiation resulted in up to 80% removal of the 3-CP after 120 hours. The striking reversal of 3-CP compared with the other derivatives may result from a bathochromic shift in the absorption of the presumed furan side monoadduct formed with the DNA. Such a shift occurs in the monoadduct analog 4',5'-dihydro-3-CP (both compounds are saturated at the 4',5' site) which has the absorption spectrum shown in figure 3. The new absorption band in the 4',5'-dihydro-3-CP occurs at approximately 360 nm, which is near the wavelength maximum of the irradiation. Thus enhanced absorption of the incident light by the 3-CP monoadduct may result in the conversion of covalently bound 3-CP back to free 3-CP followed by subsequent elimination by the photobreakdown pathway.

To investigate the nature of the noncovalent dark-bound complex, we determined the amount of DNA unwinding induced by each of the psoralen derivatives. Partially relaxed Col E1 DNA was irradiated in the presence of increasing concentrations of the derivatives. To determine the helix perturbation associated with the covalent addition of the compound, we ran agarose gel electrophoresis of the supercoiled Col E1 DNA samples. Partially relaxed DNA provides a standard series of bands, each differing by 1 superhelical turn. Any change in the superhelical density resulting from bound psoralen is detected by a shift in the mobility of the DNA bands. Unwinding of the helix relaxes the negative superhelical turns and causes the DNA to migrate more slowly. The amount of each compound covalently bound was monitored with tritium-labeled psoralen derivatives.

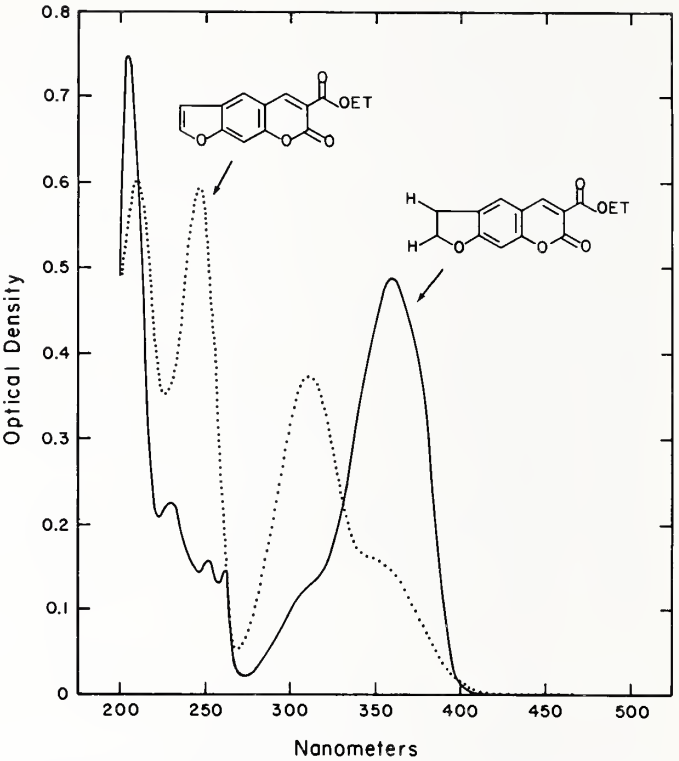


FIGURE 3.—Absorption spectra of 3-CP (.....) and 4',5'-dihydro-3-CP (———).

A typical gel is shown in figure 4. Covalent addition of increasing amounts of 8-MOP caused the DNA to migrate more slowly upon electrophoresis. The change in mobility was due primarily to unwinding of the DNA helix by the photoreacted 8-MOP. We determined the amount of unwinding by plotting the number of turns relaxed as a function of drug molecules covalently bound per Col E1 DNA molecule (fig. 5). The unwinding angles (±3°) determined for 8-MOP, 5-MOP, 3-CP, and 5-MIP were 28°, 25°, 26°, and 18°, respectively.

DISCUSSION

The dissociation constant which we determined for 8-MOP (1.4×10^{-3} *M*) is in good agreement with the previously published value of 1.3×10^{-3} *M* (*13*); it also agrees with the value of 1.36×10^{-3} *M* reported by Dall'Acqua et al. (*15*). In addition, the dissociation constant which we determined for 5-MOP (3.5×10^{-4} *M*) approximates their value of 5×10^{-4} *M* (*15*). In the same study, these authors report a dissociation constant of 7.4×10^{-4} *M* for 3-CP. Apparently, the method they used to determine the dark binding of 3-CP to DNA was spectrophotometric titration. In a separate report (*16*), a 71% decrease in the molar extinction coefficient of 3-CP upon addition of DNA was observed. We were unable to detect any decrease in the extinction coefficient of 3-CP when we

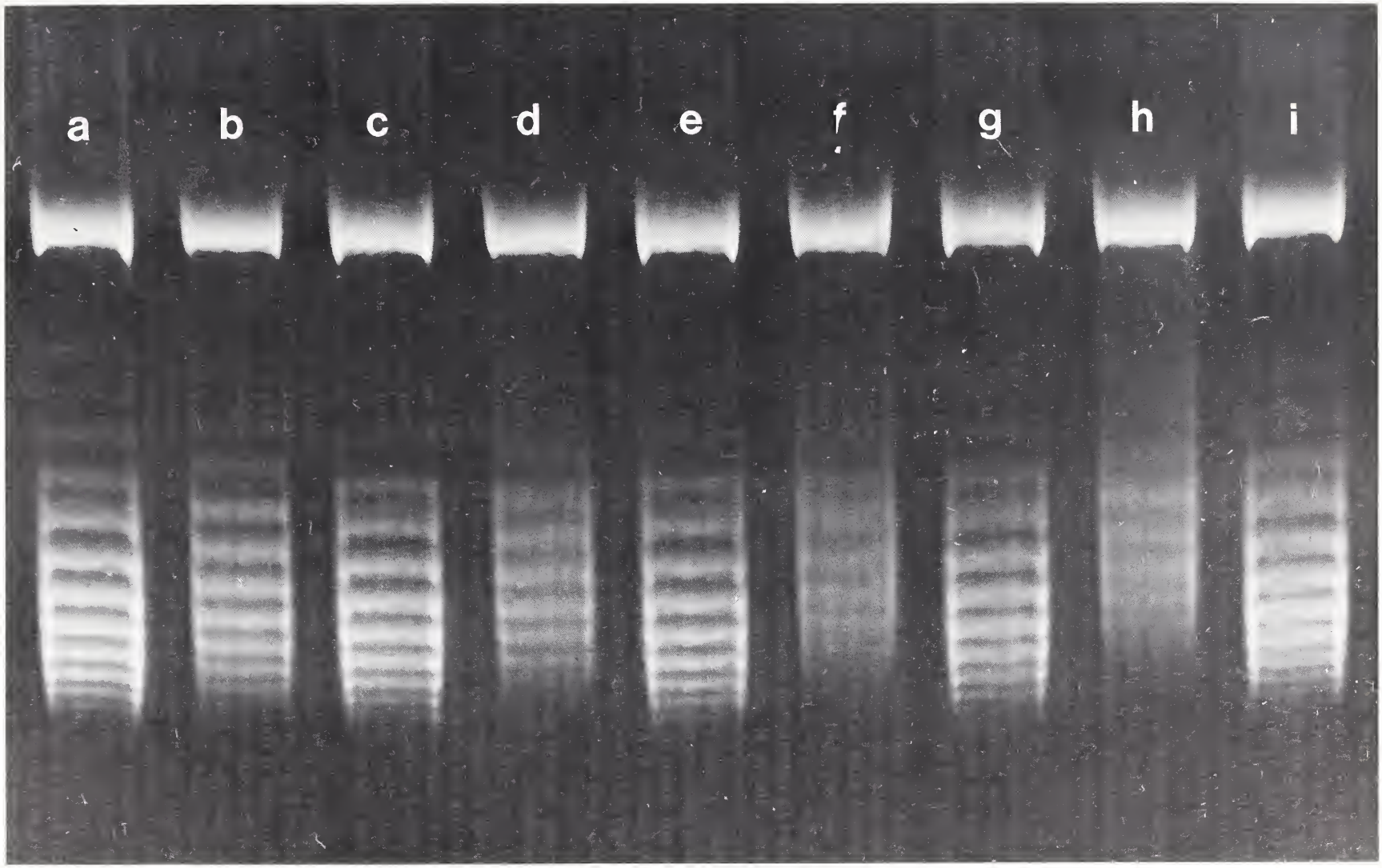


FIGURE 4.—Agarose slab gel of partially relaxed Col E1 DNA treated with 5-MIP. *Lanes:* a, c, e, g, and i, partially relaxed Col E1 DNA; b, d, f, and h, partially relaxed Col E1 DNA photoreacted with 5-MIP to the extent of 8, 16, 23, and 32 molecules of 5-MIP/molecule of Col E1 DNA, respectively.

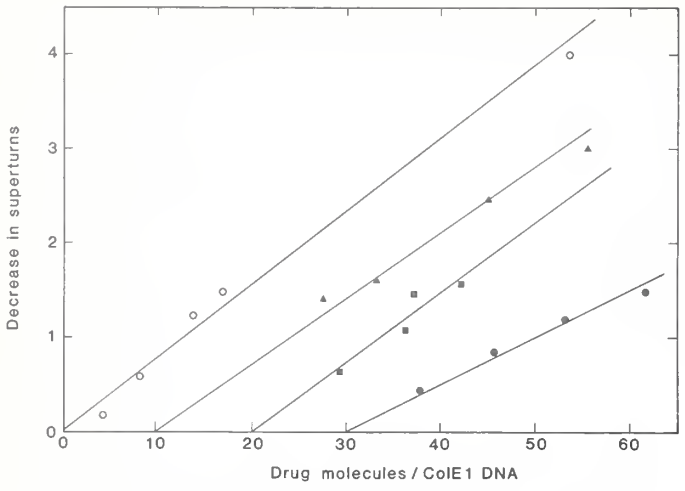


FIGURE 5.—Unwinding angles for covalent addition of 4 psoralen derivatives to Col E1 DNA. ○ = 8-MOP; the slope corresponds to 28 ± 3° unwinding/molecule reacted. ▲ = 5-MOP; the slope corresponds to 25 ± 3° unwinding/molecule reacted. Plots of this and the following 2 derivatives have been offset horizontally for clarity. ■ = 3-CP; the slope corresponds to 26 ± 3° unwinding/molecule reacted. ● = 5-MIP; the slope corresponds to 18 ± 3° unwinding/molecule reacted.

attempted a similar experiment. Thus we think our failure to observe binding of 3-CP in equilibrium dialysis was not a result of degradation of the [^{3}H]3-CP. The absence of detectable dark binding also appears to be true of our unlabeled 3-CP. The reason for the discrepancy between our results for 3-CP and those reported in (*15*) and (*16*) is unclear.

The initial rates that we found for the photoreaction of these derivatives with DNA generally agree with those found for other psoralen derivatives; 5-MIP has the highest initial rate of photoreactivity which agrees with the general observation that methylation of a psoralen usually enhances its photoreactivity. We found 8-MOP and 5-MOP to be similar in their rates of photoaddition, a result that differs from the work of Rodighiero et al. (*17*), who found that 8-MOP photoreacts 1.8 times faster than does 5-MOP. Because we are not only irradiating our solutions in a different buffer, but also using a different light source, the difference in the two sets of observations may not be significant. Our observation of low in vitro photoreactivity of 3-CP is consistent with the results of Averbeck et al. (*14*). They observed a fivefold difference in the rate of addition of 8-MOP over 3-CP and a low total amount of 3-CP bound. However, their experiments were performed in 0.1 *M* phosphate buffer (pH 7), and it is known that increased ionic strength has a strong effect on the binding constants of psoralen derivatives (*8*).

Peak values of covalent addition for 5-MIP, 5-MOP, and 8-MOP are in reasonable accord with values previously determined for other psoralen derivatives (*13*). The peak value we report for 3-CP (1 3-CP/1,000 base pairs) is one-half that reported by Averbeck and co-workers (*14*). This low DNA binding value for 3-CP correlates well with the biologic effects of 3-CP relative to 8-MOP, 5-MIP, and 5-MOP, all of which covalently bind at least thirty times more to DNA than does 3-CP (*see* table 2). Conner et al. (*18*) studied both cytotoxicity and the induction of prophage expression in 2 strains of *Salmonella typhimurium* by these 4 compounds using the identical UV light source used in this study. The induction of prophage is a component of the SOS response to DNA damage. Conner's results in these 2 assays show a striking correlation with the covalent DNA binding of the 4 compounds presented here. With both strains tested, 8-MOP, 5-MOP, and 5-MIP were highly cytotoxic, whereas comparable doses of 3-CP were much less toxic. Similarly, 8-MOP, 5-MOP, and 5-MIP were potent inducers of prophage expression, whereas 3-CP was a poor inducer of prophage. The simple argument that 3-CP is less active because it only forms monoadducts does not explain these results, inasmuch as 5-MIP, like 3-CP, likewise forms only monoadducts with DNA. We should note that the photobiologic activities of 3-CP have been reported to have a wavelength dependence (*19*). The reduced photobiologic activity of 3-CP relative to 8-MOP or 5-MOP observed by others (*20, 21*) may be explained by this wavelength dependence, or, alternatively, may reflect the low capacity of 3-CP to photoreact with DNA at any wavelength.

As mentioned above, a major difference between 3-CP and other psoralen derivatives is that it can be efficiently driven out of DNA by prolonged irradiation at 360 nm, which was also shown by Averbeck et al. (*14*). This result can be explained by the carbethoxy group presumably blocking photoaddition at the 3,4-double bond and also extending the conjugation system of the 3-CP, so that the 4′,5′-adduct has a large absorption at 365 nm. Thus the presumed 4′,5′-monoadduct can absorb a photon at 365 nm, reverse the cyclobutane ring formation, and diffuse from the DNA helix. Once the 3-CP is free in solution, it can reabsorb another photon and be degraded to a product that no longer reacts with DNA. Thus the low dark-binding constant and the high reactivity of 3-CP in solution can combine to make the peak amount of 3-CP bound low and also to allow the 3-CP which does form the monoadduct to be photoreversed. Similarly, we might expect that monoadducts of 5-MIP could be reversed, but the exact extent of reversal would depend on the dark-binding constant of the drug to DNA, the absorption of the monoadduct at 365 nm (probably much lower than that of 3-CP), and the rate of photobreakdown of drug in solution. The largest decline we observed from peak value for 5-MIP was the 15% described earlier. With a different psoralen compound, HMT, the thymidine:HMT monoadduct on the furan side of the HMT has no absorption at 360 nm (*1*). Photoreversion upon extended irradiation has not been observed with this compound; however, the HMT experiments were done with a similar but not identical irradiation source.

The unwinding angles determined in this study for 5-MOP and 8-MOP agree with those found for TMP, HMT, 4′-aminomethyl-4,5′,8-trimethylpsoralen, and 4′-methoxymethyl-4,5′,8-trimethylpsoralen (*9*). All these derivatives can form both monoadducts and cross-links with DNA. The unwinding angle per molecule that we observe is thus an average value which depends on the cross-link-to-monoadduct ratio (assuming the 2 types of adducts distort

the DNA helix by different amounts) and may also depend on the base sequence at the site of the adduct. The diadduct:monoadduct ratio for these derivatives can approach 1 (*9*). Because 5-MIP would be expected to form predominantly monoadducts, it is not surprising that it has a different average unwinding angle than do the other derivatives. Thus we conclude that the unwinding angle for an average monoadduct is about 18°, whereas that for an average diadduct may be closer to 38°.

The unwinding angle that we found was 27° for 3-CP, which is also thought to form predominantly monoadducts. This angle may be due to the bulky carbethoxy group at the 3-position that interferes with the methyl group at the 5-position in thymidine (*1*) when the 3-CP is intercalated. This steric effect may also explain the poor dark binding found with this compound.

REFERENCES

(*1*) Straub K, Kanne D, Hearst JE, et al: Isolation and characterization of pyrimidine–psoralen photoadducts from DNA. J Am Chem Soc 103:2347–2355, 1981

(*2*) Cole RS: Light-induced cross-linking of DNA in the presence of a furocoumarin (psoralen). Studies with phage λ, *Escherichia coli,* and mouse leukemia cells. Biochim Biophys Acta 217:30–39, 1970

(*3*) Musajo L, Bordin F, Caporale G, et al: Photoreactions at 3,655 Å between pyrimidine bases and skin-photosensitizing furocoumarins. Photochem Photobiol 6:711–719, 1967

(*4*) Fitzpatrick TB, Pathak MA: Historical aspects of methoxsalen and other furocoumarins. J Invest Dermatol 32:229–231, 1959

(*5*) Hönigsmann AK, Konrad K, Gschnait F, et al: Photochemotherapy of mycosis fungoides. Presented at the Seventh International Congress on Photobiology, Rome, 29 August–3 September, 1967 (Abstract)

(*6*) Parrish JA, Fitzpatrick JB, Tanenbaum L, et al: Photochemotherapy of psoriasis with oral methoxsalen and longwave ultraviolet light. New Engl J Med 291:1207–1272, 1974

(*7*) Isaacs ST, Rapoport H, Hearst JE: Synthesis of deuterium and tritium-labelled psoralens. J Labelled Compounds Radiopharm 19:345–356, 1982

(*8*) Hyde JE, Hearst JE: Binding of psoralen derivatives to DNA and chromatin: Influence of the ionic environment on dark binding and photoreactivity. Biochemistry 17: 1251–1257, 1978

(*9*) Wiesehahn G, Hearst JE: DNA unwinding induced by photoaddition of psoralen derivatives and determination of dark-binding equilibrium constants by gel electrophoresis. Proc Natl Acad Sci USA 75:2703–2707, 1978

(*10*) Clewell DB, Helinski DR: Effect of growth conditions on the formation of the relaxation complex of supercoiled Col E1 deoxyribonucleic acid and protein in *Escherichia coli.* J Bacteriol 110:1135–1146, 1972

(*11*) Modrich P, Zabel D: *Eco* R1 endonuclease. Physical and catalytic properties of the homogeneous enzyme. J Biol Chem 251:5866–5874, 1976

(*12*) Bazaral M, Helinski DR: Circular DNA forms of colicinogenic factors E1, E2 and E3 from *Escherichia coli.* J Mol Biol 36:185–194, 1968

(*13*) Isaacs ST, Chun C, Hyde JE, et al: A photochemical characterication of reactions of psoralen derivatives with DNA. *In* Trends in Photobiology (Hélène C, Charlier M, Monthenay-Garestier T, et al, eds). New York: Plenum Press 1982, pp 279–294

(*14*) Averbeck D, Moustacchi E, Bisagni E: Biological effects and repair of damage photoinduced by a derivative of psoralen substituted at the 3,4 reaction site. Photoreactivity of this compound and lethal effect in yeast. Biochim Biophys Acta 518:464–481, 1978

(*15*) Dall'Acqua F, Vedaldi D, Baccichetti F, et al: Photochemotherapy of skin diseases: Comparative studies on the photochemical and photobiological properties of various mono- and bifunctional agents. Farmaco [Sci] 36:519–535, 1981

(*16*) Gaboriau F, Vigny P, Averbeck D, et al: Spectroscopic study of the dark interaction and of the photoreaction between a new monofunctional psoralen: 3-Carbethoxypsoralen and DNA. Biochimie 63:899–905, 1981

(*17*) Rodighiero G, Musajo L, Dall'Acqua F, et al: Mechanism of skin photosensitization by furocoumarins. Photoreactivity of various furocoumarins with native DNA and with ribosomal RNA. Biochim Biophys Acta 217:40–49, 1970

(*18*) Connor M, Wheeler L, Lowe N: The induction of prophage expression in different *Salmonella typhimurium* strains by DNA cross-linking and monoadduct forming psoralens and longwave ultraviolet radiation. Carcinogenesis 4:1451–1454, 1983

(*19*) Grossweiner L, Smith K: Sensitivity of DNA repair-deficient strains of *Escherichia coli* K-12 to various furocoumarins and near-ultraviolet radiation. Photochem Photobiol 33:317–323, 1981

(*20*) Young A, Magnus I: The sunburn cell in hairless mouse epidermis: Quantitative studies with UV-A radiation and mono- and bifunctional psoralens. J Invest Dermatol 79:218–221, 1982

(*21*) Papadopoulo D, Sagliocco F, Averbeck D: Mutagenic effects of 3-carbethoxypsoralen and 8-methoxypsoralen plus 365-nm irradiation in mammalian cells. Mutat Res 124:287–297, 1983

DISCUSSION

K. Wolff: In regard to quantitation, Dr. Parrish talked about the possible significance of DNA products, cross-links, or monoadducts in the generation of erythema and the involution of psoriasis. I have a question for Mr. Isaacs. You did not give your dosages in joules. Could you correlate what really happens when you do that clinically, e.g., in photochemotherapy? What type of data would you get if you used lower doses?

S. T. Isaacs: Our data do not address that question directly. We have the intensity of the lamp in milliwatts/square centimeter, and it was approximately 4–8 mW/cm^2 from the source to the cell we were irradiating, but I would have to go back and convert that value to joules/square centimeter which we actually used. As far as comparing that to clinical results, I really cannot comment.

J. E. Hearst: We were obliged by the agency to use the lamps used clinically and at a distance comparable to that used for the patients. Presumably, the intensity we are talking about is comparable.

M. A. Pathak: Are these PUVA lamps that you used?

Isaacs: Yes.

R. Brickl: You forgot the shielding effect of the epidermis.

Hearst: That is an issue we do not wish to discuss now.

Wolff: You talked about minutes of irradiation, did you not?

Isaacs: That is correct.

Wolff: Irradiation went up to 100 minutes with 4 mW/cm^2. This amounts to tremendous doses.

Isaacs: All we were attempting to do was to compare the relative reactivity of the compounds, and as long as all the compounds were exposed to the same amount of radiation for the same amount of time, you should get a relative magnitude of binding among the compounds, and presumably that would be a linear effect. When you cut the dose, the amounts bound should drop accordingly.

Wolff: Please do not misunderstand me. What I want to get at is whether you can compare this to a clinical situation, because if you give the patient 40 joules, for instance, while he is photosensitized with 8-MOP, you are going to burn him.

Hearst: Well, I think there is no chance in the world that a patient is having psoralen added to his DNA at the level of 1 psoralen/1,000 base pairs. We use up to 20/1,000 base pairs sometimes.

Isaacs: This is given with no shielding.

Hearst: It is quite clear that doses much smaller than this are lethal to cells. Our job was to do the physical chemistry on these compounds and that is in fact what we did.

In Vitro Photoreactions of Selected Psoralens and Methylangelicins With DNA, RNA, and Proteins[1]

Giovanni Rodighiero *and* Francesco Dall'Acqua[2]

ABSTRACT—This paper presents an overview of the photophysical and photochemical properties of some furocoumarin derivatives and their interactions and photoreactions with DNA, RNA, and proteins, as well as their ability to produce singlet oxygen. The relevance of these properties in the induction of photobiologic effects is also discussed. — Natl Cancer Inst Monogr 66: 31–40, 1984.

The furocoumarin derivatives 8-MOP and TMP (*1–4*) have been used for years in the photochemotherapy of vitiligo, psoriasis, and other hyperproliferative skin diseases. More recently, 5-MOP (*5*), used diffusely in cosmetic preparations as a suntanning agent, has been investigated for its therapeutic potential.

All these furocoumarins are bifunctional psoralen derivatives; they photobind with DNA, forming both monoadducts and diadducts or cross-linkages (*6–8*). Recently, some compounds that photobind with DNA and form only monoadducts, thus behaving as monofunctional reagents, have been prepared and studied. Among these, 3-CP (*9*) and various methylangelicins, e.g., 4,5′-DMA (*10–12*), are particularly relevant for possible use in therapy for patients with psoriasis.

This paper is an overview of the photophysical and photochemical properties of these compounds (fig. 1) and their photoreactions in vitro with DNA, RNA, and proteins, as a basic contribution toward better understanding of their photobiologic and photochemotherapeutic properties.

PHOTOPHYSICAL PROPERTIES

The UV absorption and fluorescence characteristics of the considered compounds are reported in table 1; in particular, note the molar absorption coefficient at 365 nm because this radiation is the most widely used for photobiologic experiments and for psoralen and UVA therapy (*13*).

Generally, furocoumarins are highly fluorescent when exposed to UVA radiation. Remarkably, among the compounds we tested, 8-MOP has a low quantum yield of fluorescence; under the same experimental conditions, its fluorescence intensity is twenty times lower than that of psoralen (*14*).

Absorption of radiation by the furocoumarin molecules in the UVA region produces a π–π^* transition and leads to an excited species (*8*); the yield of triplet formation and the rate of decay from this excited state have been investigated for some of the compounds we tested (*15*). The yield of triplet formation is much higher in 3-CP than in 8-MOP (table 1), and it has a much longer life. This fact allows us to predict that 3-CP is a better producer of singlet oxygen than is 8-MOP (*16*).

PHOTOMODIFICATIONS

Under UVA irradiation, furocoumarins undergo various kinds of modification (for 3-CP a particular behavior has been observed; *see* below). C_4-Cyclodimers are formed that involve the 3,4-double bond of the molecules (*17, 18*). Moreover, when the compound is in solution and irradiated in the presence of oxygen, oxidation products are also formed, but they have not been thoroughly investigated. Only oxidation products resulting from a cleavage of the furanic ring have been described (*19, 20*), but probably other kinds of products are also formed. The rates of photomodification concerning dimerization and formation of oxidation products are generally low; however, remarkable differences among the various compounds have been observed. For example, after 6 hours of irradiation at 365 nm (66.5 kJ/m^2) in aqueous aerated solution, the following percentages of photomodification (including both dimers and oxidation products) were detected (*21*): 8-MOP, 6; 4,5′-DMA, 10; angelicin, 35; 8-methylpsoralen, 75; psoralen, 90. No comparable data are available for 5-MOP and TMP. Only data concerning photodimerization are available for these compounds. The rate of dimerization of 5-MOP is lower than that of psoralen and higher than that of 8-MOP (*17*); by contrast, the rate of TMP is higher than that of psoralen (*18*).

A particular reactivity has been noted for 3-CP; photomodification appears to be faster than that of the other furocoumarins. Moreover, various kinds of modifications have been detected in alcoholic solutions irradiated with 365-nm light. An addition of solvent molecules to the

ABBREVIATIONS: 8-MOP = 8-methoxypsoralen; 5-MOP = 5-methoxypsoralen; TMP = 4,5′,8-trimethylpsoralen; 3-CP = 3-carbethoxypsoralen; 4,5′-DMA = 4,5′-dimethylangelicin; UVA = UV radiation at 320–400 nm; hν = quantum of radiation; kJ = kilojoules; D_2O = deuterium oxide.

[1] Presented at the Conference on Photobiologic, Toxicologic, and Pharmacologic Aspects of Psoralens held in Research Triangle Park, North Carolina, March 1–3, 1982.

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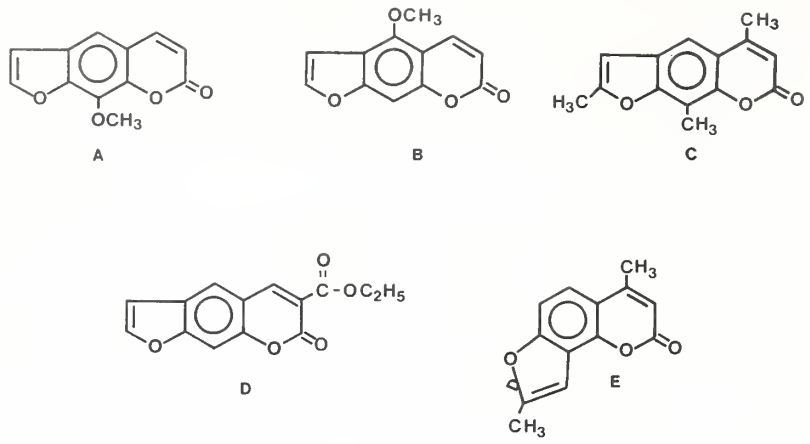

FIGURE 1.—Molecular structures of: A, 8-MOP; B, 5-MOP; C, TMP; D, 3-CP; E, 4,5′-DMA.

4′,5′-double bond and a cleavage of the furanic ring have been suggested, as well as a dimerization involving the 4′,5′-double bond (*22–24*).

PHOTOBINDING WITH FLAVIN MONONUCLEOTIDE

When aqueous solutions containing some furocoumarins and flavin mononucleotide are under irradiation at 365 nm, a covalent combination occurs between the substances resulting in compounds containing both flavinic and coumarinic moieties. Two of these less stable compounds have been isolated with 8-MOP. However, their chemical structure has not been clarified (*25*). They were lacking in the coenzymatic properties characteristic of a flavin mononucleotide (*26*), and, after acid hydrolysis, they gave rise to the mononucleotide plus a coumarinic compound deriving from an oxidative cleavage of the furanic ring of 8-MOP (*25*).

Analogous compounds, having a lower stability than but with properties similar to those isolated with 8-MOP, are also formed by 5-MOP (*19*), psoralen (*20*), and TMP (*27*). Musajo (*28*) found that photobinding does not occur by irradiation of 8-MOP with flavin adenine dinucleotide.

NONCOVALENT BINDING WITH DNA

That both linear and angular furocoumarins form noncovalent complexes with DNA deriving from intercalation of their planar molecules between 2 base pairs is well known. In the reaction

$$\text{furocoumarin} + \text{DNA} \rightleftharpoons \underset{\text{complex}}{\text{noncovalent}} \xrightarrow{h\nu} \text{covalent combination},$$

only complexed molecules photobind covalently with DNA, and the affinity of the various furocoumarins toward the macromolecule markedly affects the extent of photolesions that can be produced in DNA upon subsequent irradiation (*29*). The physicochemical parameters of the complexes formed by the tested compounds with DNA that can give precise indications on their affinity toward the macromolecule are presented in table 2 (*13*).

The behavior of the various compounds is analogous. The good inverse correlation between their water solubilities and the association constants (K values) of the complexes with DNA indicates that hydrophobic forces are mainly involved in formation of the complexes. Both K and 1/n values of the various compounds are in the same order of magnitude; TMP shows the relative highest value of the association constant, whereas 8-MOP shows the highest number of molecules intercalating in DNA.

Usually, the stability of furocoumarin–DNA complexes is low in comparison with that of other intercalating agents; therefore, no strong biologic consequences can be expected from this interaction. Until recently, only the induction, limited in some instances, of frameshift mutation in bacteria (TMP and 3-CP are inactive) has been observed (*31–33*). However, such induction occurs only at high furocoumarin concentrations and appears to be negligible in the range of concentrations occurring under therapeutic conditions (*34*).

TABLE 1.—*UV absorption and fluorescence properties*[a]

Furocoumarins	λ_{max}	ϵ_{max}	$\epsilon_{365\ nm}$	Fluorescence (alcoholic solution)[b]		Quantum yield of triplet formation in water
				Excitation	Emission	
8-MOP	302	12,014	1,150	360	460	0.06
5-MOP	312	14,200	950	350	460	
TMP	298	7,950	1,680	360	430	
4,5′-DMA	298	9,350	125	330	425	
3-CP	317	10,900	6,230	365	460	0.35

[a] Only data for the coumarinic band is presented.
[b] Excitation and emission values are given as maximum wavelengths.

TABLE 2.—*Water solubility and binding parameters of the noncovalent complexes with DNA*[a]

Furocoumarins	Water solubility		K values	n^b	$1/n^c$
	μg/ml	moles/liter			
8-MOP	23	10.60	736	7.81	0.128
5-MOP	5	2.30	2,000	15.38	0.065
TMP	1	0.45	3,970	13.33	0.075
4,5′-DMA	8	3.70	1,450	10.64	0.094
3-CP	13	5.00	1,350	28.56	0.035

[a] *See (13).*

[b] According to McGhee and von Hippel (*30*), *n* is defined as the No. of nucleotides occluded by 1 bound molecule of furocoumarin.

[c] According to McGhee and von Hippel (*30*), 1/n is defined as the frequencies of the binding sites, i.e., the No. of ligands bound/nucleotide; it can be considered similar to the "*n*" value obtained by the classic Scatchard method.

With respect to RNA, furocoumarins show a lower capacity to form a noncovalent complex because of the different and partially disordered structure of this macromolecule. Although with DNA the amount of the linked substance is practically independent of the effect of temperature in the range of 2–30° C, it is strongly temperature dependent with RNA, with higher amounts noted at low temperatures (*6*). An explanation of this different behavior may be that, although the DNA structure is poorly modified by a variation of the temperature in the above-indicated range, a decrease of the temperature with an RNA structure increases the stacking of the bases in the single-stranded portions of the molecule, and thus the possibility of complexation of the furocoumarin molecules is increased.

PHOTOBINDING WITH DNA

Under irradiation of an aqueous solution containing the DNA–furocoumarin complex, a covalent combination of the furocoumarin molecule with the macromolecule takes place. A C_4-cycloaddition occurs, in which the 5,6-double bond of pyrimidine bases (i.e., thymine or cytosine, but uracil is also reactive) is constantly involved, whereas furocoumarins generally can involve either their 3,4- or their 4′,5′-double bond. Thus 3,4- and 4′,5′-monoadducts can be formed. Because 4′,5′-monoadducts can absorb the 365-nm radiation, they may further photoreact with a second pyrimidine base, giving a diadduct. When this occurs in double-stranded DNA, a cross-linkage takes place (*6–8*).

All the furocoumarins considered here can photobind covalently with DNA. The total amounts of the various compounds (including both monoadducts and diadducts) linked to DNA as a function of time of irradiation are shown in figure 2. They have been determined recently under strictly analogous experimental conditions and are therefore comparable (*13*). From a kinetic point of view, the total photobinding always produces a pseudo first-order reaction with respect to the complexed furocoumarin, as figure 3 illustrates. The rate constant values calculated for the various compounds are as follows ($\times$ min^{-1} $\times$ 10^2):

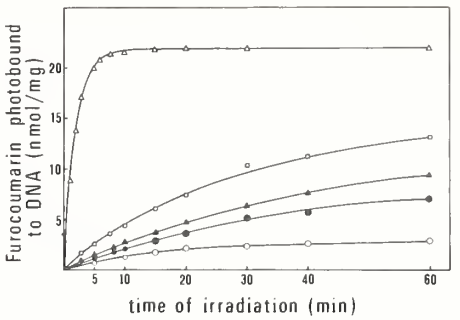

FIGURE 2.—Photobinding with DNA of the various furocoumarins as a function of time of irradiation (*13*). 8-MOP (▲), 5-MOP (●), TMP (△), 4,5′-DMA (□), 3-CP (○).

3-CP = 0.50; 5-MOP = 1.00; 8-MOP = 1.80; 4,5′-DMA = 2.30; and TMP = 53.00.

Although 8-MOP, 5-MOP, and TMP form both monoadducts and diadducts, the extent of the former is generally much higher than that of the latter photoproducts. For instance, with 8-MOP, the ratio is about 9:1. However, diadducts are most important from a biologic point of view, often having stronger consequences than monoadducts. Figure 4 shows cross-linking formation in DNA in vitro as a function of the time of irradiation under the same experimental conditions we used for studying the total photobinding.

It appears that both 4,5′-DMA and 3-CP are incapable of forming cross-linkages in DNA and form only monoadducts; thus they behave as pure monofunctional photo-

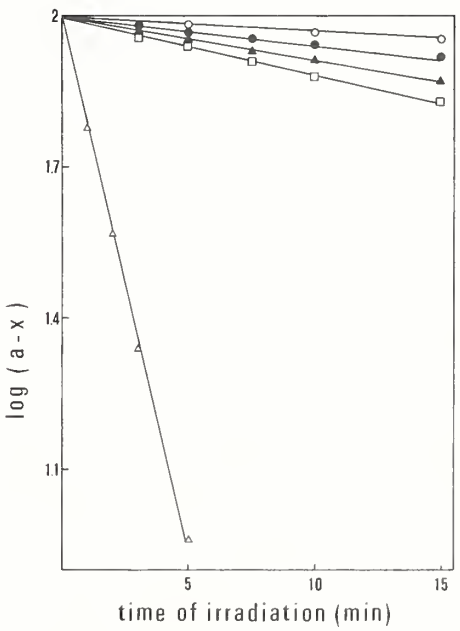

FIGURE 3.—Plot of log (a–x) against time of irradiation; a = furocoumarin complexed with DNA at zero time of irradiation; x = percent of furocoumarin in respect to that complexed with DNA, covalently photobound to the macromolecule after various times of irradiation. Symbols as in figure 2.

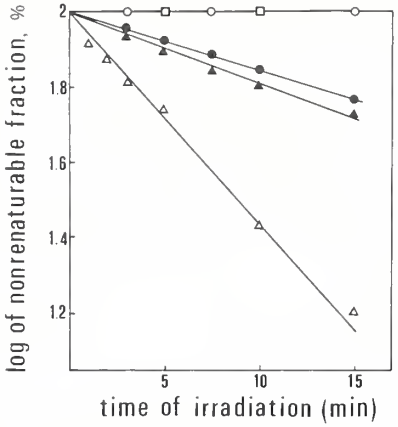

FIGURE 4.—Log of percent nonrenaturable fraction of DNA samples irradiated in the presence of various furocoumarins. Symbols as in figure 2.

reagents with DNA. Different factors may account for this property. Like all angelicin derivatives, 4,5′-DMA, when intercalated in DNA cannot assume a disposition in which its 2 photoreactive sites are suitable for photoreaction with 2 pyrimidine bases because of its angular structure (fig. 5), whereas this is possible for psoralens which have a linear structure (*35*). Only when photoreacting with the DNA of some phages that has a folded and compact structure can angelicins form cross-linkages (hairpin cross-links) between far sites of the DNA molecule that are adjacent due to the molecule's folds (*36, 37*).

On the contrary, 3-CP, although having a linear structure, contains a strong electron withdrawing group linked to the 3-position, which prevents the photoreactivity of its 3,4-double bond.

Photoreactions of furocoumarins with RNA have been much less extensively studied than have those with DNA. Generally, furocoumarins show a lower photobinding capacity with this macromolecule; moreover, the rate of photobinding is temperature dependent, whereas DNA is much less influenced by the temperature (*38*). This behavior is shown in figure 6 for the 5-MOP; similar results have been reported for 8-MOP and other linear furocoumarins (*38*). This reactivity can be easily understood, if one keeps in mind the temperature dependence of the noncovalent complexation of furocoumarins with RNA.

Some linear furocoumarins also form diadducts in photoreactions with RNA, but these actions take place between strands of 2 macromolecules (*39*).

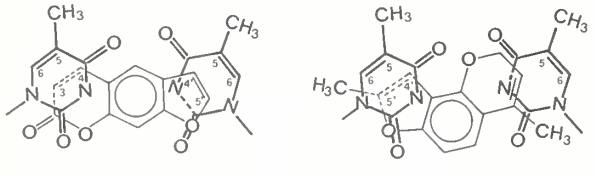

FIGURE 5.—Projection of the psoralen and 4,5′-DMA molecules intercalated between 2 base pairs of DNA. Only 2 thymines (*above* and *below* the intercalated molecule) pertaining to the opposite strands are shown.

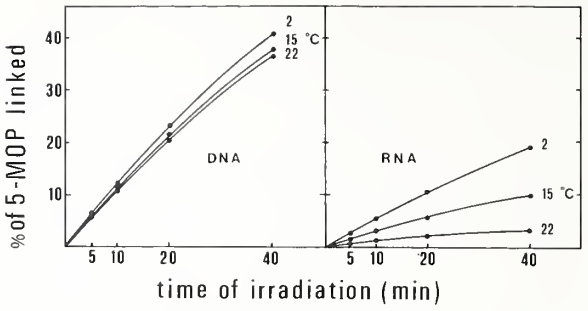

FIGURE 6.—Photobinding of 5-MOP to calf thymus DNA and to ribosomal RNA as a function of temperature and time of irradiation.

MODE OF PHOTOBINDING

The C_4-cycloaddition of furocoumarins to the pyrimidine bases of DNA has been demonstrated by the isolation of both the 3,4- and 4′,5′-monoadducts between psoralen and thymine. These have been obtained first by irradiation of an aqueous solution of the 2 compounds in different experiments (*40, 41*) and then isolated from the hydrolysis products of a DNA sample irradiated in aqueous solution in the presence of a psoralen (*42*). They have also been found in DNA of Ehrlich ascites tumor cells irradiated in the presence of a psoralen (*43*). Similar monoadducts have also been obtained from 5-MOP (*40*).

More 3,4-monoadducts are formed in the presence of a psoralen than are 4′,5′-monoadducts (*44*); for the psoralen derivatives we investigated, no exact evaluations are available. On the bases of some properties (e.g., the fluorescence acquired by DNA after photoaddition), we can assume that both 3,4- and 4′,5′-monoadducts are always formed.

Recently, the 4′,5′-monoadduct with thymine has been isolated from a sample of DNA irradiated in the presence of 4,5′-DMA (*45*). The 3,4-monoadduct has also been isolated, but under different experimental conditions, i.e., from an irradiated mixture of 4,5′-DMA and thymine (Dall'Acqua F, Caffieri S, Vedaldi D, et al: Unpublished results). They demonstrated that in DNA 95% of 4,5′-DMA is linked through its 4′,5′-position.

Because 3-CP forms only monofunctional adducts and its 3,4-double bond is prevented from photoreacting by the presence of the carbethoxy group, one can assume that it photoreacts only by means of its 4′,5′-double bond. However, the structures of the photoproducts originated have not been definitely clarified. They appear to be different from those of the monoadducts formed by other furocoumarins. Fluorescence studies have indicated that an opening of the 4′,5′-double bond is involved in the photocombination (*46*).

PREFERRED SITES IN DNA

Several investigators have examined polydeoxyribonucleotides with known repeating sequences to determine whether the interactions between furocoumarins and DNA occur randomly along the macromolecule or at the level of preferred sequences. Various DNA samples of different

TABLE 3.—*Noncovalent interaction between some furocoumarins and human serum albumin: binding parameters of the complexes and percentages of the bound furocoumarins*[a]

Furocoumarins	Binding parameters of the complexes with human serum albumin		Percent bound fraction[b]	
	$K_a \times 10^{-4}$	n	Human serum albumin	Human serum
Psoralen	2.28	1.8	89	94
8-MOP	1.27	2.4	84	90
5-MOP	3.80	1.65	91	93
8-Methylpsoralen	1.70	1.6	92	94
TMP	19.10	1.6	97	98
Angelicin	1.90	1.0	85	88

[a] *See* (*54*).

[b] Data were obtained by equilibrium dialysis at 37° C. Furocoumarin concentration = 1 μg/ml; human serum albumin concentration = 30 mg/ml in 0.05 *M* phosphate buffer, pH 7.4, 0.9% sodium chloride.

origins and base compositions have been studied both for noncovalent complex formation and covalent photobinding of some furocoumarins.

The reported results (*14, 47–50*) indicate that furocoumarins do not intercalate randomly but preferentially in regions having an alternate sequence of purines and pyrimidines without showing a preference between adenine–thymine and cytosine–guanine.

These types of regions are also preferred for covalent photobinding, with a specific preference, in this case, for the alternate sequence of adenine–thymine. For cross-link formation, an alternate sequence of adenine–thymine and cytosine–guanine seems to be preferred.

These results, obtained with various linear furocoumarins such as 8-MOP (*14*), have recently been confirmed with the angular furocoumarin 4,5′-DMA and with 5-methylangelicin (*50*). We can assume that they may be extended to the other furocoumarin derivatives now being studied.

INTERACTIONS WITH PROTEINS

Recently, studies have been done on the noncovalent binding between furocoumarins and proteins and the possible covalent linkage between these substances.

Data on the noncovalent complex formation between some furocoumarins and serum albumin are shown in table 3 (*51, 52*). Furocoumarins have a strong affinity for serum albumin as shown in the high value for TMP. Only a small number of furocoumarin molecules are linked per albumin molecule. For psoralen derivatives having a linear structure this number is nearly 2 (1.6–2.4), but for angelicin with an angular structure it is 1. These small numbers and the relatively high values of the association constants clearly suggest that serum albumin has specific binding sites for furocoumarins.

This specificity is true only for serum albumin. With other proteins, such as thermolysine, ribonuclease, and chimotrypsin, the percentages of binding found for psoralen are much lower or close to zero (*53*). Therefore, the structures of proteins (probably due to the presence of highly hydrophobic sites) and furocoumarins are fundamentally important for the formation of a molecular complex involving noncovalent bonds.

A covalent photobinding of furocoumarins with proteins may occur, but only with extremely low yields under irradiation with 365-nm light (*53, 54*). The extent of linkage is different for the various furocoumarins, depending also on the nature of the proteins, as is shown by the data in table 4 and from those reported for 8-MOP by Yoshikawa et al. (*54*). At present, 2 hypotheses can be suggested concerning the mechanisms through which photobinding takes place, based on our results obtained when we irradiated a furocoumarin in the presence and absence of oxygen, or preirradiated it, and then added it to the protein solution (table 4): 1) addition of the furocoumarin molecule to an amino acid of the protein molecule (perhaps through reactive radical intermediates), and 2) photooxidation of the furocoumarin molecule into new reactive intermediates, which form the covalent linkage with proteins. Probably the 2 mechanisms occur concomitantly when a protein solution is irradiated in the presence of a furocoumarin (*53*); however, this premise must be verified.

TABLE 4.—*Covalent photobinding of furocoumarins with some proteins; irradiation with 365 nm for 6 hr (66.5 kJ/m²)*[a]

Furocoumarins	Protein-bound furocoumarins (moles furocoumarins/mole protein)					
	Bovine serum albumin[b]			Thermolysine[c]	Ribonuclease[c]	Chimotrypsin[c]
	Following irradiation under O_2	Following irradiation under N_2	Following preirradiation under O_2			
Psoralen	2.10	0.90	0.60	0.50	2.00	1.2
8-MOP	0.33	0.25	0.11	0.10	0.60	
Angelicin	0.35					
4,5′-DMA	0.20	0.15		0.02	0.08	0.03

[a] *See* (*55*).

[b] The molar ratio of furocoumarins to protein in the irradiated solution was 5:1.

[c] Substances were irradiated in the presence of oxygen. The molar ratio of furocoumarins to protein in the irradiated solution was 30:1.

SINGLET OXYGEN PRODUCTION

The possible formation of singlet oxygen during the irradiation of 8-MOP with 365-nm light by energy transferred from the triplet excited state of 8-MOP to the molecular oxygen has been demonstrated by Poppe and Grossweiner (*55*). The singlet oxygen thus produced is highly reactive and oxidizes the biologic substrates with which it comes in contact:

$$\text{8-MOP} \xrightarrow{h\nu} \text{8-MOP}^*$$
$$\text{8-MOP}^* + O_2 \longrightarrow \text{8-MOP} + O_2^*$$
$$O_2^* + \text{substrate} \longrightarrow \text{oxidation products.}$$

This reaction has been verified also for other furocoumarin derivatives, such as 5-MOP, TMP, and angelicin. The extent of singlet oxygen produced (which is always low) varies for the different compounds. However, in this respect, the results reported by various authors are not in agreement because of the different methods used for its determination. According to Cannistraro and Van de Vorst (*56*), activity decreased in the following order: 5-MOP> psoralen>8-MOP and angelicin>5-hydroxypsoralen and 8-hydroxypsoralen (inactive). De Mol and Beijersbergen van Henegouwen (*57*) present the order as TMP> psoralen>8-MOP>5-MOP>5,8-dimethoxypsoralen; they found angelicin inactive. The last order of activity, which is the same as that for the induction of erythema on guinea pig skin, allowed the authors to suggest that singlet oxygen formation is involved in the production of skin erythema.

No direct evidence has been reported for 4,5′-DMA; its inability to inactivate enzymes suggests that its capacity to produce singlet oxygen is lower than that of other furocoumarins.

The previously mentioned high capacity of 3-CP to form the triplet excited state, much higher than that of 8-MOP, suggests that this compound produces considerable singlet oxygen (*16*). This suggestion is confirmed by the fact that some of its photobiologic properties (e.g., its lethal effect on *Saccharomyces cerevisiae*) are more pronounced in the presence than in the absence of oxygen and by the higher oxidative effect exerted on some amino acids, compared with other furocoumarin derivatives (*58*).

Laser flash photolysis applied to 3-CP complexed with DNA has not detected any formation of a triplet excited state of the molecule. Although photogeneration of singlet oxygen may be expected by the noncomplexed molecules, it is unlikely when they are intercalated in DNA (*16*). Also, Poppe and Grossweiner (*55*) reported that the singlet oxygen production by 8-MOP is quenched by the presence of polynucleotides which form a complex with it. By contrast, De Mol et al. (*59*) reported that 5-MOP produces singlet oxygen even when complexed with DNA.

Further studies are necessary for clarification of the various discrepancies in this field and for evaluation of the role of singlet oxygen in inducing the photobiologic effects of furocoumarins.

PHOTOINACTIVATION OF ENZYMES

Irradiation of some enzymes in the presence of furocoumarins may produce inactivation of their properties (*59–62*). Large doses of radiation are necessary; the extent of inactivation is different depending on the furocoumarin structure and on the enzymatic nature, as is shown in table 5. From the results reported, 4,5′-DMA and 8-MOP have little capacity to photoinactivate enzymes, except lysozymes, whereas 3-CP exerts a much higher activity.

Recalling what has been previously reported, one may question whether inactivation is due to the covalent binding of furocoumarins or to the oxidation of some amino acids produced by the singlet oxygen generated by furocoumarins, or both. If both reactions are responsible, the problem may be that their relative importance must be evaluated.

Veronese et al. (*62*) found that amino acid analyses performed on lysozyme and glutamate dehydrogenase, partially inactivated by irradiation in the presence of tritiated psoralen, revealed that at least 5 and 7 amino acids were modified per molecule of enzyme. A modification occurred for the following amino acids: methionine (recovered as methionine sulfoxide), tyrosine, phenylalanine, histidine, and tryptophan. However, radioactivity measurements indicated that only 0.8 and 0.9 molecule of psoralen was covalently linked to 1 molecule of lysozyme or glutamate dehydrogenase, respectively. Therefore, the modification of amino acids in these enzymes was due only in small part to the covalent photobinding of psoralen. This finding stresses the importance of the singlet oxygen produced by the furocoumarins during the irradiation for the inhibition of the enzymatic activity. Other experiments, such as the irradiation of enzymes in a D_2O solution or, by contrast, in the presence of singlet oxygen quenchers, confirmed this conclusion (*62*).

TABLE 5.—*Photoinactivation of enzymes in the presence of furocoumarins with different structures*[a]

Furocoumarins	Percent activity after 3 hr UVA irradiation (33.25 kJ/m^2)					
	Glutamate dehydrogenase	6-Phosphogluconate dehydrogenase	Lysozyme	Enolase	Ribonuclease	Thermolysine
Psoralen	40	33	32	100	100	
8-Methylpsoralen	60	10	62	100	70	85
8-MOP	98	98	70	95		
Angelicin	75	95	82	95		
4,5′-DMA	100	98	98	98		98
3-CP	29	13	26	21	75	

[a] *See* (*62*).

CONCLUSIONS

The photochemical properties of furocoumarins can be summarized by the following scheme, which indicates the ways by which a furocoumarin molecule, excited by absorption of a photon in the UVA region, can dissipate the absorbed energy:

Furocoumarin$^{(*)}$
- Fluorescence and phosphorescence emission
- Dimerization
- Photobinding with flavin mononucleotide
- Photobinding with pyrimidine bases of DNA
- Photobinding with pyrimidine bases of RNA
- Photobinding with proteins
- Energy transfer producing singlet oxygen

Under suitable conditions, all these events can occur at the same time. Therefore, from a theoretical point of view, all the indicated photochemical reactions and energy transfer may be important for the induction of photobiologic effects.

The importance of a photochemical process derives not only from the biologic significance of the involved substrate but also from the rate of occurrence of the process. Therefore, using data available in the literature, we tried to calculate the relative extent of the various processes to obtain indications of their relevance. Although the available data refer to different experimental conditions, a rough comparison was possible concerning the photomodification and the photobinding with DNA, RNA, and proteins for 8-MOP (table 6).

For the production of singlet oxygen, the data we found in the literature did not allow the calculation of parameters directly comparable with those reported in table 6. Considering the large amounts of radiation generally used for measuring the singlet oxygen produced by 8-MOP and other furocoumarins in vitro, we believed the quantum yield was extremely low.

The data presented in table 6, although only indicative, allow us to conclude that the most sensitive photoreaction of 8-MOP is the photobinding with DNA. Most likely, this is also true for other furocoumarins; however, due to their different properties, the results shown in table 6 for 8-MOP may be different when other furocoumarins are considered.

TABLE 6.—*Relative amounts of UVA radiation necessary for the various photoreactions of 8-MOP*

Photoreactions	Relative amounts resulting from the calculations referring to the linkage of 5 nmol of 8-MOP to:	
	1 mg of macromolecule	1 mmol of macromolecule
DNA (calf thymus)	1	1
RNA (ribosomal)	10	120
Proteins (bovine serum albumin)	6.5	610
Photomodifications[a]	4	

[a] Reference is to the modification (including both dimerization and formation of oxidation products) of 5 nmol of 8-MOP.

REFERENCES

(1) EL MOFTY AM: Vitiligo and Psoralens. Oxford: Pergamon Press, 1968

(2) FITZPATRICK TB, PARRISH JA, PATHAK MA: Phototherapy of vitiligo (idiopathic leukoderma). *In* Sunlight and Man: Normal and Abnormal Photobiologic Responses (Fitzpatrick TB, Pathak MA, Harber LC, et al, eds). Tokyo: Univ Tokyo Press, 1974, pp 783–791

(3) PARRISH JA, FITZPATRICK TB, TANENBAUM L, et al: Photochemotherapy of psoriasis with oral methoxsalen and longwave ultraviolet light. N Engl J Med 291: 1207–1211, 1974

(4) ANDERSON TF, VOORHEES JJ: Psoralen photochemotherapy of cutaneous disorders. Annu Rev Pharmacol Toxicol 20:235–257, 1980

(5) HÖNIGSMANN H, JASHKE E, GSCHNAIT F, et al: 5-Methoxypsoralen (bergapten) in photochemotherapy of psoriasis. Br J Dermatol 101:369–377, 1979

(6) MUSAJO L, RODIGHIERO G: Mode of photosensitizing action of furocoumarins. *In* Photophysiology (Giese AC, ed), vol VII. New York: Academic Press, 1972, pp 115–147

(7) PATHAK MA, KRÄMER DM, FITZPATRICK TB: Photobiology and photochemistry of furocoumarins (psoralens). *In* Sunlight and Man: Normal and Abnormal Photobiologic Responses (Fitzpatrick TB, Pathak MA, Harber LC, et al, eds). Tokyo: Univ Tokyo Press, 1974, pp 335–368

(8) SONG P-S, TAPLEY KJ: Photochemistry and photobiology of psoralens. Photochem Photobiol 29:1177–1197, 1979

(9) DUBERTRET L, AVERBECK D, ZAJDELA F, et al: Photochemotherapy (PUVA) of psoriasis using 3-carbethoxypsoralen, a non-carcinogenic compound in mice. Br J Dermatol 101:379–389, 1979

(10) BORDIN F, CARLASSARE F, BACCICHETTI F, et al: 4,5′-Dimethylangelicin: A new DNA-photobinding monofunctional agent. Photochem Photobiol 29:1063–1070, 1979

(11) DALL'ACQUA F, VEDALDI D, GUIOTTO A, et al: Methylangelicins: New potential agents for the photochemotherapy of psoriasis. Structure–activity studies on the dark and photochemical interactions with DNA. J Med Chem 24:806–811, 1981

(12) PATHAK MA, FITZPATRICK TB: Certain biological and therapeutic properties of linear and angular furocoumarins (psoralens and isopsoralens). Presented at the Ninth Annual Meeting of the American Society for Photobiology, Williamsburg, Va., 1981 (Abstract TAM E-1)

(13) DALL'ACQUA F, VEDALDI D, BACCICHETTI F, et al: Photochemotherapy of skin diseases: Comparative studies on the photochemical and photobiological properties of various mono- and bifunctional agents. Farmaco [Sci] 36:519–535, 1981

(14) DALL'ACQUA F, VEDALDI D, BORDIN F, et al: New studies on the interaction between 8-methoxypsoralen and DNA in vitro. J Invest Dermatol 73:191–197, 1979

(15) BENSASSON RV, LAND EJ, SALET C: Triplet excited states of furocoumarins: Reaction with nucleic acid bases and amino acids. Photochem Photobiol 27:273–280, 1978

(16) DUBERTRET L, AVERBECK D, BENSASSON R, et al: Photophysical, photochemical, photobiological and photochemotherapeutic properties of 3-carbethoxy-psoralen. *In* Psoralens in Cosmetics and Dermatology (Cahn J, Forlot P, Grupper C, et al, eds). Oxford, New York: Pergamon Press, 1981, pp 245–256

(17) RODIGHIERO G, CAPPELLINA V: Ricerche sulla fotodimerizzazione di alcune furocumarine. Gazz Chim Ital 91: 103–114, 1961

(18) MARCIANI S, DALL'ACQUA F, RODIGHIERO P, et al:

Ricerche sulla foto-C_4-ciclo-dimerizzazione di alcuni metilpsoraleni. Gazz Chim Ital 100:435–451, 1970

(*19*) MUSAJO L, RODIGHIERO G, CAPORALE G, et al: La fotoreazione tra bergaptene e flavin-mononucleotide. Gazz Chim Ital 94:1054–1072, 1964

(*20*) RODIGHIERO G, MUSAJO L, DALL'ACQUA F, et al: La fotoreazione tra psoralene e flavin-mononucleotide. Gazz Chim Ital 94:1073–1083, 1964

(*21*) VERONESE FM, SCHIAVON O, BEVILACQUA R, et al: The effect of psoralens and angelicins on proteins in the presence of UV-A irradiation. Photochem Photobiol 34:351–354, 1981

(*22*) VIGNY P, GABORIAU F, DUQUESNE M, et al: Spectroscopic properties of psoralen derivatives substituted by carbethoxy-groups at the 3,4 and/or 4′,5′ reaction site. Photochem Photobiol 30:557–564, 1979

(*23*) LANGLOIS E, MUEL B, BISAGNI E, et al: Photolysis of 3-carbethoxypsoralen in alcoholic solutions. Presented at the Eighth International Congress on Photobiology, Strasbourg, July 20–25, 1980 (Abstract P 169)

(*24*) MULLER-RUNKEL R, GROSSWEINER LI: Dark membrane lysis and photosensitization by 3-carbethoxypsoralen. Photochem Photobiol 33:399–402, 1981

(*25*) RODIGHIERO G, MUSAJO L, FORNASIERO U, et al: La fotoreazione tra xantotossina e flavin-mononucleotide. Gazz Chim Ital 94:1084–1092, 1964

(*26*) AZZONE GF, RODIGHIERO G, MUSAJO L, et al: Ricerche enzimologiche sui flavin foto-composti. Gazz Chim Ital 94:1101–1107, 1964

(*27*) CAPORALE G, RODIGHIERO G, GIACOMELLI C, et al: Le fotoreazioni tra flavin-mononucleotide e metilpsoraleni. Gazz Chim Ital 95:513–552, 1965

(*28*) MUSAJO L: Photoreactions between flavin-coenzymes and skin-photosensitizing agents. Pure Appl Chemistry 6: 369–384, 1963

(*29*) DALL'ACQUA F: Photochemical reactions of furocoumarins. *In* Trends in Photobiology (Hélène C, Charlier M, Monthenay-Garestier T, et al, eds). New York: Plenum Press, 1981, pp 267–278

(*30*) MCGHEE JD, VON HIPPEL PH: Theoretical aspects of DNA–protein interactions: Cooperative and noncooperative binding of large ligands to one-dimensional homogeneous lattice. J Mol Biol 86:469–482, 1974

(*31*) CLARK CH, WADE MJ: Evidence that caffeine, 8-methoxypsoralen and steroidal dimers are frameshift mutagens for *E. coli* K 12. Mutat Res 28:123–125, 1975

(*32*) ASHWOOD-SMITH MJ: Frameshift mutations in bacteria produced in the dark by several furocoumarins; absence of activity of 4,5′,8-trimethylpsoralen. Mutat Res 58:23–27, 1978

(*33*) BRIDGES BA, MOTTERSHEAD RP: Frameshift mutagenesis in bacteria by 8-methoxypsoralen (methoxsalen) in the dark. Mutat Res 44:305–312, 1977

(*34*) BRIDGES BA: An estimate of genetic risk from 8-methoxypsoralen and steroidal dimers are frameshift mutagens 1979

(*35*) DALL'ACQUA F, MARCIANI S, CIAVATTA L, et al: Formation of inter-strand cross-linkings in the photoreactions between furocoumarins and DNA. Z Naturforsch [B] 26:561–569, 1971

(*36*) KITTLER L, HRADEČNA Z, SUHNEL J: Cross-link formation of phage lambda DNA in situ photochemically induced by the furocoumarin derivative angelicin. Biochim Biophys Acta 607:215–220, 1980

(*37*) BACCICHETTI F, BORDIN F, CARLASSARE F, et al: T_2 phage sensitization by linear and angular furocoumarins. Z Naturforsch [C] 34:811–814, 1979

(*38*) DALL'ACQUA F, MARCIANI S, RODIGHIERO G: Photoreactivity (3,655 Å) of various skin-photosensitizing furocoumarins with nucleic acids. Z Naturforsch [B] 24:307–314, 1969

(*39*) MARCIANI S, TERBOJEVICH M, DALL'ACQUA F, et al: Bifunctional photobinding of psoralen to single-stranded nucleic acids. Z Naturforsch [C] 28:370–375, 1973

(*40*) MUSAJO L, BORDIN F, CAPORALE G, et al: Photoreactions at 3,655 Å between pyrimidine bases and skin-photosensitizing furocoumarins. Photochem Photobiol 6:711–719, 1967

(*41*) MUSAJO L, BORDIN F, BEVILACQUA R: Photoreactions at 3,655 Å linking the 3,4-double bond of furocoumarins with pyrimidine bases. Photochem Photobiol 6:927–931, 1967

(*42*) MUSAJO L, RODIGHIERO G, DALL'ACQUA F, et al: Prodotti di fotocicloaddizione a basi pirimidiniche isolati da DNA idrolizzato dopo irradiazione a 3655 Å in presenza di psoralene. Rend Accad Naz Lincei (Rome) 42:457–468, 1967

(*43*) MUSAJO L, BORDIN F, BACCICHETTI F, et al: Psoralen–thymine C_4-cycloadduct formed in the photoinactivation with psoralen of Ehrlich ascites tumor cells. Rend Accad Naz Lincei (Rome) 43:442–447, 1967

(*44*) DALL'ACQUA F, MARCIANI MAGNO S, ZAMBON F, et al: Kinetic analysis of the photoreaction (365 nm) between psoralen and DNA. Photochem Photobiol 29:489–495, 1979

(*45*) DALL'ACQUA F, CAFFIERI S, VEDALDI D, et al: Monofunctional 4′,5′-photocycloadduct between 4,5′-dimethylangelicin and thymine. Photochem Photobiol 33:261–264, 1981

(*46*) GABORIAU F, VIGNY P, AVERBECK D: Comparative study of 3-carbethoxypsoralen and 8-methoxypsoralen: Dark interaction and photoreaction with DNA. Presented at the Eighth International Congress on Photobiology, Strasbourg, July 20–25, 1980 (Abstract P 179)

(*47*) MARCIANI S, DALL'ACQUA F, VEDALDI D, et al: Receptor sites of DNA for the photoreaction with psoralen. Farmaco [Sci] 31:140–151, 1976

(*48*) DALL'ACQUA F, TERBOJEVICH M, MARCIANI S, et al: Investigation on the dark interaction between furocoumarins and DNA. Chem Biol Interact 21:103–115, 1978

(*49*) DALL'ACQUA F, VEDALDI D, RECHER M: The photoreaction between furocoumarins and various DNA with different base compositions. Photochem Photobiol 27: 33–36, 1978

(*50*) VEDALDI D, RODIGHIERO P, GUIOTTO A, et al: Receptor sites in DNA for the dark and photochemical interactions with 4,5′-dimethylangelicin, a potential agent for the photochemotherapy. Chem Biol Interact 36:275–286, 1981

(*51*) VERONESE FM, BEVILACQUA R, SCHIAVON O, et al: The binding of 8-methoxy-psoralen by human serum albumin. Farmaco [Sci] 33:667–675, 1978

(*52*) ———: Drug–protein interaction: Plasma protein binding of furocoumarins. Farmaco [Sci] 34:3–12, 1979

(*53*) VERONESE FM, SCHIAVON O, BEVILACQUA R, et al: The effect of psoralens and angelicins on proteins in the presence of UV-A radiation. Photochem Photobiol 34: 351–354, 1981

(*54*) YOSHIKAWA K, MORI N, SAKAKIBARA S, et al: Photoconjugation of 8-methoxypsoralen with proteins. Photochem Photobiol 29:1127–1133, 1979

(*55*) POPPE W, GROSSWEINER LI: Photodynamic sensitization by 8-methoxypsoralen via the singlet oxygen mechanism. Photochem Photobiol 22:217–219, 1975

(*56*) CANNISTRARO S, VAN DE VORST A: ESR and optical absorption evidence for free radical involvement in the

photosensitizing action of furocoumarin derivatives and for their singlet oxygen production. Biochim Biophys Acta 476:166–177, 1977

(*57*) De Mol NJ, Beijersbergen van Henegouwen GM: Relation between some photobiological properties of furocoumarins and their extent of singlet oxygen production. Photochem Photobiol 33:815–819, 1981

(*58*) Averbeck D, Averbeck S, Dall'Acqua F: Mutagenic activity of three monofunctional and three difunctional furocoumarins in yeast (*Saccharomyces cerevisiae*). Farmaco [Sci] 36:492–505, 1981

(*59*) De Mol NJ, Beijersbergen van Henegouwen GM, van Beele B: Singlet oxygen formation by sensitization of furocoumarins complexed with, or bound covalently to DNA. Photochem Photobiol 34:661–666, 1981

(*60*) Pathak MA, Fellman JH: Photosensitization by furocoumarins (psoralens). *In* Progress in Photobiology (Christensen BC, Buchmann B, eds). Amsterdam, New York: Elsevier, 1961, pp 552–554

(*61*) Veronese FM, Schiavon O, Bevilacqua R, et al: Drug–protein interaction: 8-Methoxypsoralen as photosensitizer of enzymes and amino acids. Z Naturforsch [C] 34: 392–396, 1979

(*62*) Veronese FM, Schiavon O, Bevilacqua R, et al: Photoinactivation of enzymes by linear and angular furocoumarins. Photochem Photobiol 36:25–30, 1982

DISCUSSION

K. Smith: Unless I misunderstood, I thought Dr. Rodighiero said that 3-CP was a good singlet oxygen generator and Dr. Pathak said it was not.

G. Rodighiero: The formation of singlet oxygen by 3-CP is not a direct result of my experiments. This assumption comes from its ability to give a high quantum yield of the triplet state and from the long life of its triplet state. We must remember that the formation of singlet oxygen occurs by energy transfer from the triplet state of a sensitizer.

Moreover, Dr. Averbeck demonstrated that some photobiologic properties of 3-CP are oxygen dependent, i.e., they are higher in the presence of oxygen than in its absence. Furthermore, the photoinactivation of some enzymes is produced to a much higher extent by 3-CP than by other furocoumarin derivatives. However, I must point out that I do not know if this inactivation is produced by singlet oxygen formation or by a higher combination of 3-CP with the proteins.

This furocoumarin has a particular behavior under irradiation: It is less stable than other furocoumarins, and it forms radical intermediates, which may produce linkages to these proteins to a higher extent than do other furocoumarins.

P-S. Song: Singlet oxygen production depends on two factors. One is, as Dr. Rodighiero mentioned, intersystem crossing yield or triplet yield. The other is the energy level; the energy transfer through singlet oxygen is unspecific, so what determines it are the relative energy gaps between the donor triplet and the singlet oxygen level. All the psoralens, as I indicated, have virtually identical triplet levels. So that means that with psoralens only one pathway determines the singlet oxygen production yield. In fact, the higher triplet yield should result in higher singlet oxygen yield.

L. Grossweiner: I agree, but do you have much higher photochemical decomposition?

Song: There is one factor that would significantly raise the effect of triplet yield in vivo and that is once a chromophore binds to a macromolecule, e.g., proteins, their triplet yields are drastically affected. If for some reason isopsoralens are trapped by proteins and other macromolecules, they may show a much lower triplet yield in the in vivo skin conditions and are thus not able to produce singlet oxygen. That is a possibility.

D. Averbeck: I just want to add to this. We were much puzzled by this question of the oxygen effect. In eukaryotic cells, 3-CP shows a strong oxygen effect, whereas this was not observed with 8-MOP. Our finding appeared to correspond well to the triplet quantum yields of these 2 compounds. Therefore, we believe that, at least in this system, the cellular sensitization in the presence of oxygen is important with 3-CP. We do not see this with 8-MOP, although we know that in in vitro conditions, singlet oxygen is produced by 8-MOP.

M. Pathak: How stable is 3-CP?

Averbeck: It is not highly stable, but it is stable enough so that molecules can photobind to cellular DNA before they are destroyed.

Pathak: Then the reactive moiety must be the problem.

Averbeck: Dr. Isaacs showed a destruction curve of 3-CP. In fact, even in biologic experiments we have seen that preirradiation of 3-CP in solution with high doses gave rise to its total destruction but no biologic effect of 3-CP was observed.

S. Isaacs: The literature seems to indicate that the compound breaks down readily in solution. Is that right?

Averbeck: Yes, it does. One has to be extremely careful. Especially in the presence of light, we usually talk about small doses.

Grossweiner: When we studied the photosensitized inactivation of lysozyme by 3-CP and compared it with 8-MOP, we found that 3-CP was a much more active photosensitizer for lysozyme inactivation, but oxygen was protective. The sensitization was much more effective under nitrogen. The kinetics of that reaction were indicative of an externally generated inactivating species, which led us to conclude that it was the radicals and not singlet oxygen that was inactivating the enzyme. I think it is an open question as to whether 3-CP is an active singlet oxygen generator because of the high competition from possible radical reactions.

Averbeck: We did another experiment using D_2O, an agent which prolongs the triplet state of singlet oxygen. In the presence of 3-CP and D_2O, we observed a further increase in sensitivity of the cells. If one thinks singlet oxygen is really there, this would be a good indication.

Grossweiner: What was the factor of increase?

Averbeck: It was quite substantial but less than the difference we obtained between the response under oxygen and under nitrogen.

Grossweiner: Was there evidence that in the D_2O triplet state lifetimes are increased as well?

Averbeck: Yes.

Grossweiner: I assume that it was not as large as the lifetime of singlet oxygen. I think that a D_2O effect has to be quite substantial before it is taken as sufficient evidence that singlet oxygen is involved.

Averbeck: Well, we did this experiment under nitrogen, and, in the presence of D_2O, we saw an increase in sensitivity, but this increase in sensitivity was not as large as under oxygen.

Mechanisms of Psoralen Photosensitization Reactions[1, 2]

Madhu A. Pathak[3]

ABSTRACT—The basic aspects of cutaneous photosensitization reactions and the mode of therapeutic effectiveness of psoralens used extensively in the photochemotherapy of psoriasis and vitiligo have been reviewed. Psoralen-induced skin photosensitization and the therapeutic action of psoralens involve two distinct types of reactions that occur independently of each other and concurrently when the psoralen-treated skin (oral or topical) is exposed to 320–400 nm UV radiation. The first, type I, occurs in the absence of oxygen (anoxic) reaction and primarily involves photoreactions with DNA; the second, type II, is a sensitized reaction dependent on oxygen and involves the formation of singlet oxygen ($^1O_2$), superoxide anion (O_2^-), and hydroxy radicals. The photoreactive form of psoralen is its triplet state, and the sites of reaction are 1) the cell membrane of the epidermal, dermal, and endothelial cells; 2) the cytoplasmic constituents, such as enzymes, RNA, lysosomes, etc.; 3) the cell nuclei (DNA and chromatin); and 4) psoralen-sensitized production of $^1O_2$, O_2^-, and hydroxy radicals, which we believe are responsible for cell membrane damage and vasodilation. The major damage would be initiated by a type I reaction and would be seen in the form of nuclear damage to DNA resulting from the interaction of psoralen with DNA and to a lesser extent with RNA. The skin photosensitization response (erythema, edema, membrane damage, etc.) would result from a type II reaction involving the generation of $^1O_2$. In vitro and in vivo metabolism of 4,5′,8-trimethylpsoralen and 8-methoxypsoralen is briefly outlined. — Natl Cancer Inst Monogr 66: 41–46, 1984.

PHOTOREACTIVE STATES OF PSORALENS

Furocoumarins are heterocyclic aromatic compounds derived from the condensation of a furan ring with a coumarin ring. Although this fusion may occur in several ways (e.g., in 12 linear and nonlinear or angular forms), only 2 forms of furan and coumarin fusion are generally seen and have been studied extensively: 1) a linear, tricyclic structure resembling psoralen, and 2) a nonlinear, angular structure called angelicin or isopsoralen (fig. 1).

Abbreviations: 8-MOP = 8-methoxypsoralen; TMP = 4,5′,8-trimethylpsoralen; 5-MOP = 5-methoxypsoralen; ^1Ps = singlet excited state of psoralen; ^3Ps = triplet state of psoralen; Ps_0 = deactivated or ground-state psoralen; $^1O_2$ = singlet oxygen; O_2^- = superoxide anion; DMCP = 4,8-dimethyl,5′-carboxypsoralen.

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[2] Supported in part by Public Health Service grant 2-R01-CA05003 from the National Cancer Institute.

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Until recently, only linear psoralens, such as 8-MOP, TMP, psoralen, and 5-MOP, were used in the photochemotherapy of vitiligo and psoriasis. The angular psoralens (angelicins or isopsoralens) were considered of no therapeutic value but are now being examined for their biologic and therapeutic effectiveness. Some of the important properties of linear and angular psoralens are summarized in table 1. Linear psoralens capable of forming interstrand cross-links with DNA and also of producing either $^1O_2$, O_2^-, or free radicals are generally potent skin photosensitizing agents that produce erythema, edema, increased pigmentation, and even a vesiculation reaction (*1*). Nonlinear or angular psoralens usually do not form interstrand cross-links with DNA; they are less reactive to form $^1O_2$ or free radicals and are considered to be nonphotosensitizing agents.

Linearly annulated psoralens (e.g., 8-MOP, TMP, and 5-MOP) and nonlinear or angular psoralens (e.g., angelicin or isopsoralen and 5-methylangelicin) exhibit well-defined UV absorption spectra with several distinct absorption peaks. The first peak is in the tail end of the UVA region ($>$ 300 to 335 nm); the second and third absorption peaks are in the UVB and UVC regions (245–295 nm, 210–220 nm, respectively). The absorption band at 200 to 335 nm is attributable to $\pi \longrightarrow \pi^*$ electron transition. It is now well known that the action spectra of 8-MOP and several other reactive furocoumarins for eliciting the skin photosensitization response (erythema or increased pigmentation) are in the long-wavelength UV region extending from 320 to 380 nm, with maximum effectiveness in the 330- to 340-nm band (*2, 3*). Upon absorption of 320–400 nm radiation, the psoralen molecules are converted to a new, unstable, but electronically excited, reactive state that is short-lived (10^{-8} sec). This reactive state is called the "singlet excited state" because the paired electrons in their molecular orbits spin in opposite directions (*1, 3, 4*). From this ^1Ps, the electrons of the photoreactive psoralens undergo an intersystem crossing (or an electronic spin flip) in which one electron changes in spin to acquire a parallel spin. Such a state is called the "triplet state" (^3Ps); it is short-lived (10^{-3} second or longer) but significantly longer lasting and probably more capable of reacting than singlet-excited state psoralen. During this conversion of ^1Ps to ^3Ps, some absorbed energy is lost. Consequently, ^3Ps have a lower energy level than do singlet-excited (^1Ps) states. Although photoexcited psoralens are reactive in both the ^1Ps and ^3Ps, the longer lifetime of the latter state, however, permits it to react with other biologic molecules with greater probability than the molecule in ^1Ps. From the short-lived ^1Ps (10^{-8} second) and the long-lived ^3Ps (10^{-3} second), three

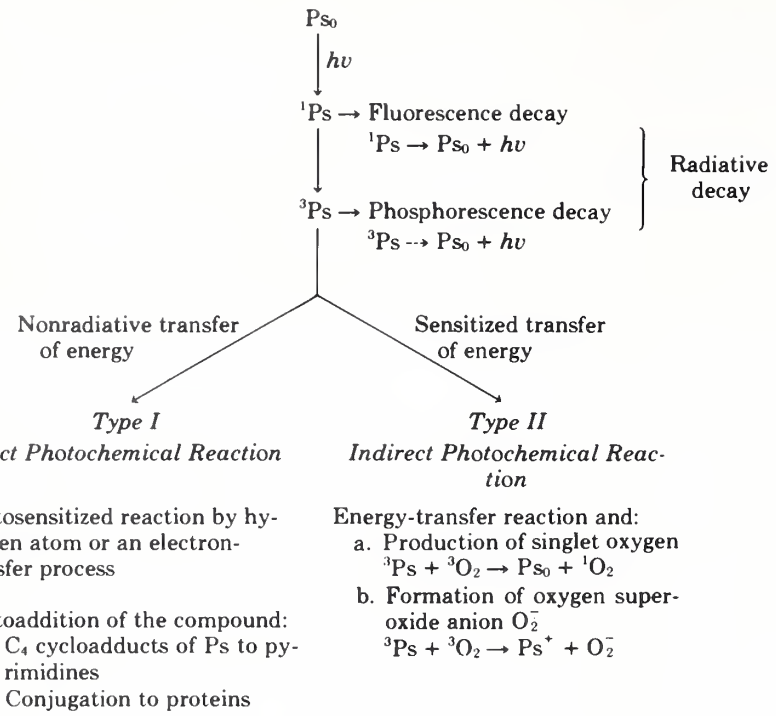


FIGURE 1.—Photoexcited states of psoralen and their in vitro and in vivo photochemical reactions. Two types of reactions are shown: type I, anoxic and involving nonradiative transfer of energy, and type II, oxygen dependent, involving sensitized transfer of energy to molecular oxygen.

events tend to occur: 1) radiative decay, 2) nonradiative transfer of energy (type I reaction), and 3) sensitized transfer of energy (type II reaction) in which the energy of ^3Ps is transferred to oxygen ($^3O_2$) with the formation of $^1O_2$, O_2^-, and free radicals.

In the first process, the ^1Ps becomes deactivated and returns to its Ps_0 by a process known as "radiative decay" (fig. 1). From ^1Ps, the absorbed radiation is emitted in the form of fluorescence, and from the ^3Ps it is emitted in the form of phosphorescence. Generally, no photochemical or photosensitization event results from this radiative decay process.

In the second process, the nonradiative transfer of energy from ^3Ps to biologic substrates (the so-called anoxic or type I reaction), the ^3Ps psoralen causes certain specific chemical reactions in vivo in which the excited state directly reacts with the biologic substrates. These reactions include: 1) the cyclobutane addition of psoralen to pyrimidine bases, giving monofunctional C_4 adducts (both fluorescent and nonfluorescent); 2) the interstrand cross-linking of psoralen

TABLE 1.—*Properties of monofunctional and bifunctional psoralens*

Property	Bifunctional	Monofunctional
Molecular structure	Mostly linear	Mostly nonlinear, exception 3-carbethoxypsoralen
Skin photosensitizing activity	Strongly phototoxic	Absent, nonerythemogenic[a]
Ability to form: $^1O_2$	Strong	Moderate to high
O_2^-	Moderate	Moderate to strong
Free radicals	Strong	Weak to moderate
Stimulation of melanin pigmentation	Strong, ++ to ++++	Weak, + to ++
Mutagenicity	Strong, ++ to ++++	Moderate to strong, + to +++
Carcinogenicity	Topical, strong	Topical, moderate to high[b]
	Oral, weak	Oral, not investigated[b]
DNA intercalation	Strong	Weak to moderate
Photobinding capacity to DNA	"	Weak to moderately high
Monoadduct-forming capacity with DNA	"	Strong
Interstrand cross-linking property with DNA	"	Absent
Inhibition of DNA and RNA synthesis	"	Moderate to high
Therapeutic effectiveness in psoriasis	Strong and well investigated	Weak to moderate and not extensively investigated
Therapeutic effectiveness in other diseases (vitiligo, mycosis fungoides, etc.)	Strong and effective	Not investigated

[a] Some isopsoralens, especially methyl-substituted angelicin derivatives, have been recently found to produce skin photosensitization (erythema, edema, and vesiculation) in our laboratory, both in human volunteers and guinea pigs.

[b] 3-Carbethoxypsoralen, which is noncarcinogenic topically and orally, is an exception.

to pyrimidine bases of the opposite strand of DNA; 3) the photoreaction of psoralens with amino acids of proteins (e.g., keratin and albumin) and with lipoproteins of cell membrane; and 4) the photochemical alteration of psoralens (e.g., dimerization of psoralen).

In the third process, the sensitized transfer of energy (type II reaction), the damage to DNA, proteins, and cell membrane result from reactions with species other than the photosensitizing compound itself. This involves the formation of reactive oxygen species ($^1O_2$, O_2^-, and hydroxy radicals). The interaction of these reactive oxygen species with biologic substrates produces photooxidized molecules. These photooxidative reactions, commonly referred to as "photodynamic reactions," occur only in the presence of molecular oxygen. These reactions are schematically shown in figure 1.

PHOTOREACTIONS OF PSORALENS AND DNA

The photoreaction between psoralens and DNA can be divided into 4 distinct steps: 1) ground state complexation of psoralen in the dark with DNA bases (the formation of noncovalent complexes with DNA), involving intercalation of psoralen between adjacent base pairs in the DNA molecule; 2) photoreaction between the 4′,5′-double bond of the furan ring of a psoralen molecule and the 5,6-double bond of the pyrimidine base (e.g., thymine) to form a cyclobutane (C_4) adduct referred to as a "fluorescent monoadduct"; 3) photoreaction of the 3,4-double bond of the pyrone ring of psoralen and the 5,6-double bond of pyrimidine base to form a cyclobutane adduct referred to as a "nonfluorescent monoadduct"; and 4) the absorption of a second photon by the fluorescent monoadduct to yield an interstrand cross-link (fig. 2). This involves the conversion of fluorescent monoadducts to interstrand cross-links between the 2 pyrimidine bases belonging to opposite strands of DNA (*3–8*). The interstrand cross-links are believed to be largely responsible for eliciting the photosensitizing action of psoralens in mammalian skin. Those psoralens which form interstrand cross-links are known to cause erythema, edema, vesiculation, and increased pigmentation. Monofunctional psoralens which do not form cross-links but only form either the fluorescent or nonfluorescent adducts with DNA do not cause skin photosensitization in the form of erythema, but they do evoke damage to DNA and cell membranes and increased pigmentation of skin. Isopsoralen and angelicin usually form only monofunctional adducts. Many linear psoralens (e.g., 3-carbethoxypsoralen, 4′,5′-dihydropsoralen) also form only monofunctional adducts.

Several investigators (*1, 3, 5*) have suggested that psoralens forming bifunctional adducts (cross-links) are capable of skin photosensitization and those forming only monofunctional adducts are nonphotosensitizing and do not evoke erythema. However, this general rule is not strictly valid; exceptions are gradually emerging, and we have seen bifunctional (interstrand cross-linking) psoralens that do not evoke skin photosensitization, e.g., 8-hydroxypsoralen, 5,8-dimethoxypsoralen, 4,8-dimethyl-5′-carboxypsoralen (*1*).

Recent observations indicate that certain monofunctional

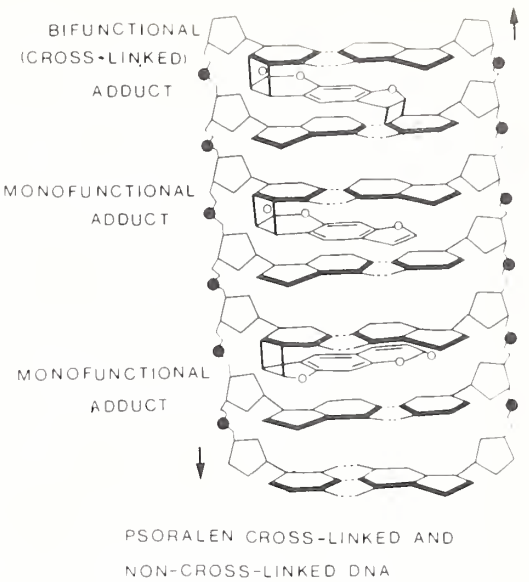


FIGURE 2.—Photoconjugation of psoralen with DNA showing the formation of 2 types of monofunctional adducts (fluorescent and nonfluorescent) with pyrimidine bases and bifunctional (interstrand cross-links) adducts in DNA.

psoralens, such as 5-methylangelicin and 4,6,4′-trimethylangelicin, are as photosensitizing as bifunctional 8-MOP or TMP (Pathak MA: Unpublished observations). They induce erythema, edema, and increased skin pigmentation.

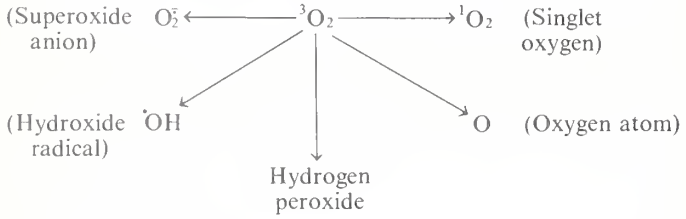
The formation of monofunctional and bifunctional photoadducts in DNA results in the immediate inhibition of DNA synthesis. Subsequently, cell proliferation is also inhibited in patients with psoriasis who receive repeated PUVA treatment. The production of these psoralen–DNA adducts is a function of the dose of the drug administered, the dose of the UV exposure, and the wavelength of irradiation. With linear psoralens, irradiation with wavelengths of 320 to 360 nm generally evokes monofunctional and bifunctional adducts, whereas irradiation with longer wavelengths (380–420 nm) generally results in the formation of monofunctional adducts. Excessive production of these cyclobutane adducts causes cell death. Those cells which survive DNA damage and undergo DNA replication tend to repair this damage through an error-prone repair process that eventually appears to be responsible for the carcinogenic effects of psoralens on skin. The phenomena of cell death, mutation, and skin carcinogenesis apparently result from the photoconjugation of psoralens to DNA.

HISTORICAL BACKGROUND AND PRESENT CONCEPTS OF SKIN PHOTOSENSITIZATION BY PSORALENS

The combined use of psoralens and UV radiation for the treatment of vitiligo was introduced in the early 1950s (*1*). In those days, the science of photomedicine was in its infancy. Most of the photosensitized reactions examined were shown to require the participation of molecular oxygen (*9*) and were often termed "photodynamic actions" involving oxygen, visible light, and photooxidation reactions. In the early 1960s, photosensitizing chemicals were

classified into 2 categories on the basis of their requirement for oxygen: 1) oxygen-dependent reactions, in which the sensitizer was not often consumed in the reaction, e.g., many dyes, such as eosin, rose bengal, and methylene blue (*9*), and 2) anoxic or oxygen-independent reactions, such as the photoreactions involving furocoumarin or psoralen, in which molecular oxygen was not required for action and the sensitizer was more often consumed in the reaction (*10–12*). Subsequently, photobiologists advanced the concepts of electronically excited molecules (e.g., ^{1}Ps and ^{3}Ps) and the field of oxygen-dependent photodynamic reactions by which the sensitizers interacted with oxygen to produce oxidized molecules and biologic damage (*3, 4, 11, 13*). The mode of reaction of photosensitizing dyes was better understood with the realization that a relatively long-lived $^1\Delta_g$ state of $^1O_2$ was generated in the photosensitized reactions (*13–16*).

In many photosensitization reactions it is now recognized that $^1O_2$, O_2^-, hydroxy radicals, and hydrogen peroxide are formed. Interconversion of these reactive species is also possible and is schematically shown below (*13–16*):



With the realization that electronically excited singlet molecular oxygen has a relatively long lifetime (from microseconds to milliseconds) and that it can diffuse and have accessibility to cell membranes, its biologic role in elucidating the psoralen-induced skin photosensitization reaction became apparent (*15, 17*). Investigators then realized that in vivo psoralen photosensitization could occur both in the presence and absence of oxygen (*17, 18*). The photoreactions of psoralens with skin DNA would represent a type I reaction (anoxic reaction), whereas the photodamaging reaction of psoralen with endothelial cells and cell membranes would be a type II reaction (oxygen-dependent photodynamic action). In photosensitization reactions, the absorption of photons by such cellular constituents as DNA, RNA, proteins, enzymes, lipoproteins, etc., either directly (type I reaction) or through the energy-transfer process (type II reaction) results in the injury or damage to the nuclei, organelles, membranes, and enzymes of cells. Clinically, these oxygen-dependent photodynamic reactions of skin would be manifested in the form of exaggerated sunburn, edema, vesiculation, and subsequent desquamation and hyperpigmentation (or even occasionally as depigmentation resulting from loss of melanocytes). The arterioles, venules, and capillaries, which often are damaged in the photosensitized reaction of skin, contain the highest concentration of molecular oxygen. Formation of $^1O_2$ or O_2^-, through the sensitized reaction is thus an event contributing to endothelial cell damage and subsequent vasodilation. Among the various excited forms of molecular oxygen ($^1O_2$, O_2^-, and hydroxy radicals), $^1O_2$ is of particular physiologic importance because of its selectively long life in aqueous solutions and its ability to diffuse to distances greater than 10 angstroms. By the end of 1982, it became clear that most photosensitized reactions of psoralens involved excited electronic states, usually triplet states of the sensitizer molecule (*1, 4, 13, 17, 18*). The excited sensitizer either reacted directly with the substrate (e.g., photoaddition of psoralen to DNA) or alternatively with some other molecule in the reaction mixture (e.g., oxygen), giving reactive oxygen ($^1O_2$), which in turn reacted with a substrate. The anoxic or type I reaction was the predominant one. Psoralen reactions thus appear to be complex and involve both the type I and type II reaction pathways, as illustrated in figure 1.

MODES OF PSORALEN PHOTOSENSITIZATION

The exact mechanism by which psoralens produce cutaneous photosensitivity reactions is not known, but a great deal of progress has been made in elucidation of its nature.

The manner by which psoralens cause cellular injury and produce adverse cutaneous responses (erythema, edema, vesiculation, hyperpigmentation, etc.) can best be understood if we focus on the fact that the mode of action of psoralens does not occur on one level but on several levels simultaneously.

On the basis of our present knowledge and supporting experimental evidence, one could state that in normal skin or in the skin of psoriatic patients, psoralen-induced skin photosensitization involves 2 distinct types of reactions that occur independently of each other and concurrently when the psoralen-treated skin (oral or topical) is exposed to 320- to 400-nm radiation. Type I is an oxygen-independent reaction, and type II is a sensitized reaction dependent on oxygen and the formation of $^1O_2$, O_2^-, and free radicals. In these 2 modes of reactions, the reactive form of psoralen is in its triplet state, and the sites of reaction are: 1) the cell membrane of the epidermal, dermal, and endothelial cells; 2) cytoplasmic constituents (enzymes, RNA, lysosomes, etc.); 3) cell nuclei (DNA and chromatin); and 4) the photodynamic reaction involving the production of $^1O_2$, O_2^-, or hydroxy radicals, and these reactive forms would induce the oxidation in lipids present in the lipoprotein membrane of the cell. The damage caused by the photoexcited psoralen present on the cell membrane could be manifested in the form of cellular edema commonly seen in the psoralen photosensitization reaction. These reactive oxygen species, in turn, can cause membrane damage to arterioles and venules and may contribute to the formation of secondary oxidation products that lead to the increased formation of prostaglandins and prostacyclines.

The major photochemical reaction contributing to cellular damage is undoubtedly the formation of monofunctional and bifunctional (interstrand cross-links) adducts in DNA and is a type I reaction. The skin photosensitization response manifested in the form of erythema and edema would most likely be due to the generation of $^1O_2$ or O_2^- (type II reaction), or both, resulting from the interaction of psoralen (^{3}Ps) with oxygen ($^3O_2$). Because $^1O_2$ and O_2^- are highly reactive forms of oxygen, the formation of these reactive moieties of oxygen can also explain the damage to

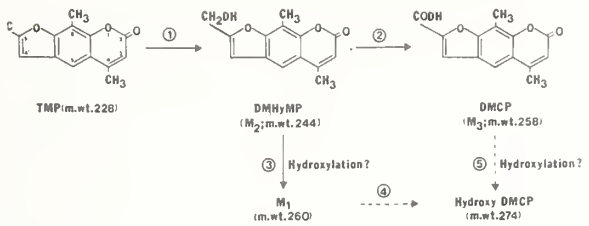


FIGURE 3.—Metabolism of TMP in humans and mice. Reactions 1, 2, and 3 were confirmed in vitro. Products DMCP and hydroxy-DMCP were isolated from human and mouse urine after TMP was administered orally. m. wt. = molecular weight. Figure is reproduced with permission of the publisher (*21*).

DNA, the induction of erythema, and its inhibition by $^1O_2$ and O_2^- quenchers, e.g., sodium azide and superoxide dismutase, etc., damage to the cell membrane, and the edema reaction.

METABOLISM OF 4,5′,8-TRIMETHYLPSORALEN AND 8-METHOXYPSORALEN

Since 1974, 8-MOP has been recognized as the most effective psoralen in the photochemotherapy of psoriasis and other skin diseases such as vitiligo, mycosis fungoides, etc. Little is known of its biotransformation and of the enzymes involved in its metabolism. The nature of urinary metabolites excreted by humans or laboratory animals receiving oral TMP and 8-MOP have been studied in our laboratory, and these findings are summarized below. (Experimental details of this research will be published elsewhere.)

Mandula and associates (*19, 20*) reported the isolation and identification of a major metabolite (DMCP) and hydroxy-DMCP from the urine of human volunteers and mice receiving oral TMP. A metabolic pathway integrating in vivo and in vitro TMP reactions in mice and humans is shown in figure 3. Reactions marked as 1, 2, and 3 were established in vitro with the use of mouse microsomal fractions and liver homogenates under mixed function oxidase conditions. Both DMCP and hydroxy-DMCP have been synthesized and their properties examined (*21*).

By contrast, 8-MOP apparently is minimally metabolized in vitro by the mouse liver homogenates or microsomal fractions. Guinea pig epidermis also shows no metabolic transformation of either 8-MOP or TMP. However, when 5-[^{14}C]8-MOP is administered to mice orally, over 60% is excreted in the urine within 12 hours in the form of 7 metabolites (4 major, 3 minor). Chemical structures tentatively assigned to the 4 major metabolites, M-2, M-3, M-4, and M-7, are shown in figure 4. These are: 8-Hydroxypsoralen (labeled as A) is M-3; the epoxide of 8-MOP and its dihydrodiol (labeled as B and C) are referred to as M-2 and are shown in 2 forms. Furocoumaric acid (labeled as D) is M-4, an open chain lactone that can cyclize to form an M-7 metabolite labeled as metabolite E (8-MOP); the structures of the other metabolites designated as M-1, M-5, and M-6 are not shown. We determined that M-1 exhibited an absorption spectrum with a characteristically broad absorption band between 280 and 290 nm and an absorption peak at 330 nm. Unlike 8-MOP, M-1 shows no absorption peaks between 240 and 280 nm but does show a steep rise in the absorption value between 200 and 230 nm.

The major metabolite M-2 appears to have 3 possible structures which are labeled B, C, and F in figure 4. Detectable in the urine of humans and mice, M-2 shows UV absorbance peaks at 217 and 320 nm, a fluorescence emission peak at 510 nm, and a photoexcitation peak at 313 nm. This metabolite exhibits weak but definite phototoxicity reactions in guinea pig skin and shows the varying molecular weights of 232 and 250. The mass spectra of M-2 revealed a molecular weight of 232 or 250. The molecular weights of metabolites B, C, and F are consistent with their assigned structures. The fluorescent property of this M-2 metabolite and the varying nature of its molecular weight suggests that it is an epoxide of 8-MOP that becomes

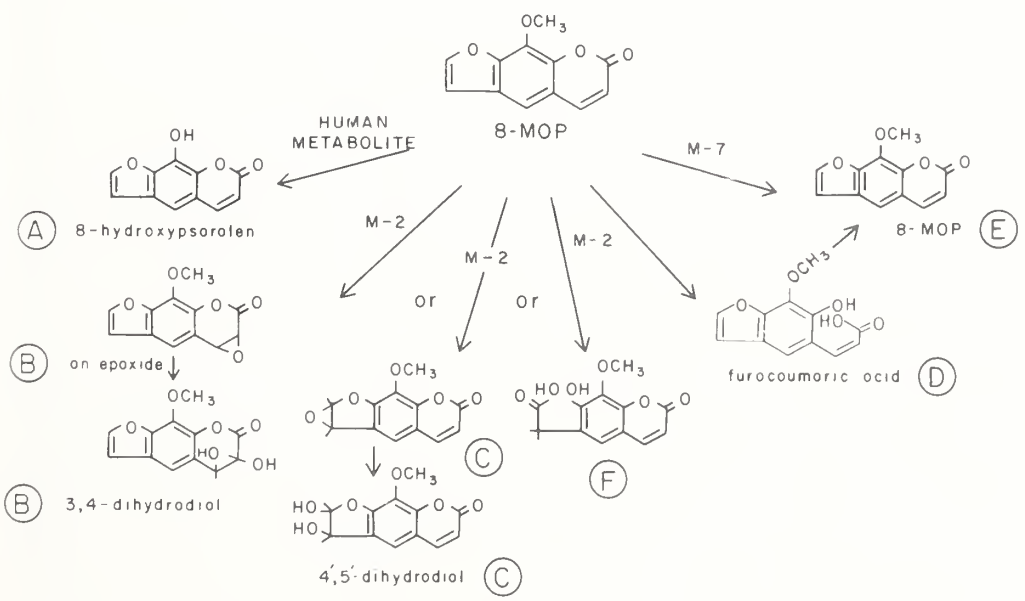


FIGURE 4.—Metabolic pathway integrating in vitro and in vivo 8-MOP metabolism. Identification of these metabolites, their absorption spectra, molecular weights, and other biochemical and biophysical characteristics will be published elsewhere. Figure is reproduced with permission of the publisher of *J Invest Dermatol* 79:201–205, 1982.

converted to a dihydrodiol; both compounds are intensely fluorescent. The epoxidation and its conversion to a hydroxylated moiety appears to be at the 4′,5′-double bond; thus M-2 most likely appears to be 8-methoxy,4′,5′-dihydriol psoralen (*see* C in fig. 4). The most likely metabolite of 8-MOP, involving the formation of 8-hydroxypsoralen (A in fig. 4) through demethylation, could not be detected in mouse urine but could be seen in that of human volunteers who received oral 8-MOP. In addition, one of the fluorescent metabolites appeared to be an open-chain furocoumaric acid moiety of 8-MOP (D in fig. 4). The 8-hydroxypsoralen and furocoumaric acid were nonphototoxic to the skin and induced no photosensitization reaction when they were applied topically and the skin was subsequently irradiated (320–400 nm). The metabolite M-7, exhibiting 217-, 248-, and 303-nm absorption peaks, was isolated as a glucuronide and, upon hydrolysis, was identified as 8-MOP with a molecular weight of 216. Human volunteers receiving 0.6–1.2 mg 8-MOP/kg orally revealed urinary excretion of 4 fluorescent metabolites, of which the prominent were M-2, 8-MOP, furocoumaric acid, and a metabolized 8-MOP in the form of a glucuronate.

REFERENCES

(*1*) PATHAK MA, JOSHI PC: The nature and molecular basis of cutaneous photosensitivity reactions to psoralens and coal tar. J Invest Dermatol 80 (Suppl):66s–74s, 1983

(*2*) NAKAYAMA Y, MORIKAWA F, FUKUDA M, et al: Monochromatic radiation and its application. Laboratory studies in the mechanism of erythema and pigmentation induced by psoralen. *In* Sunlight and Man: Normal and Abnormal Photobiologic Responses (Fitzpatrick TB, Pathak MA, Harber LC, et al, eds). Tokyo: Univ Tokyo Press, 1974, pp 591–611

(*3*) PATHAK MA, KRÄMER DM, FITZPATRICK TB: Photobiology and photochemistry of furocoumarins (psoralens). *In* Sunlight and Man: Normal and Abnormal Photobiologic Responses (Fitzpatrick TB, Pathak MA, Harber LC, et al, eds). Tokyo: Univ Tokyo Press, 1974, pp 335–368

(*4*) PATHAK MA: Molecular aspects of drug photosensitivity with special emphasis on psoralen photosensitization reaction. JNCI 69:163–170, 1982

(*5*) MUSAJO L, RODIGHIERO G, CAPORALE G, et al: Photoreactions between skin photosensitizing furocoumarins and nucleic acids. *In* Sunlight and Man: Normal and Abnormal Photobiologic Responses in Man (Fitzpatrick TB, Pathak MA, Harber LC, et al, eds). Tokyo: Univ Tokyo Press, 1974, pp 369–387

(*6*) POPPE W, GROSSWEINER LI: Photodynamic sensitization by 8-methoxypsoralen via the singlet oxygen mechanism. Photochem Photobiol 22:217–219, 1975

(*7*) DALL'ACQUA F, MARCIANI S, ZAMBON F, et al: Kinetic analysis of the photoreaction (365 nm) between psoralen and DNA. Photochem Photobiol 29:489–495, 1979

(*8*) SONG P-S, TAPLEY KJ JR: Photochemistry and photobiology of psoralens. Photochem Photobiol 29:1177–1197, 1978

(*9*) BLUM HF: Photodynamic Action and Disease Caused by Light. ACS Monogr Ser, No. 85. New York: Reinhold, 1941

(*10*) FOWLKS WL: The mechanism of the photodynamic effect. J Invest Dermatol 32:233–247, 1959

(*11*) SANTAMARIA L, PRINO G: The photodynamic substances and their mechanisms of action. *In* Research Progress in Organic, Biological, and Medicinal Chemistry (Gallo U, Santamaria L, eds), vol 1. Milan: Societá Editoriale Farmaceutica, 1964, pp 260–336

(*12*) SPIKES JD, LIVINGSTON R: The molecular biology of photodynamic action: Sensitized photoautooxidation in biological systems. Adv Radiat Biol 3:29–121, 1969

(*13*) TURRO NJ: Modern Molecular Photochemistry. San Francisco: Benjamin, 1977

(*14*) FOOTE CS: Photosensitized oxidation and singlet oxygen: Consequences in biological systems. *In* Free Radicals in Biology (Pryor WA, ed), vol 2. New York: Academic Press, 1976, pp 85–133

(*15*) ITO T: Cellular and subcellular mechanisms of photodynamic action: The $^1O_2$ hypothesis as a driving force in recent research. Photochem Photobiol 28:493–508, 1978

(*16*) SINGH A: Introduction: Interconversion of singlet oxygen and related species. Photochem Photobiol 28:429–433, 1978

(*17*) PATHAK MA, JOSHI PC: Production of active oxygen species ($^1O_2$ and O_2^-) by psoralens and ultraviolet radiation (320–400 nm). Biochim Biophys Acta 798:115–126, 1984

(*18*) JOSHI PC, PATHAK MA: Production of singlet oxygen and superoxide radicals by psoralens and their biological significance. Biochem Biophys Res Commun 111:638–646, 1983

(*19*) MANDULA BB, PATHAK MA, DUDEK G: Photochemotherapy: Identification of a metabolite of 4,5′,8-trimethylpsoralen. Science 193:1131–1135, 1976

(*20*) MANDULA BB, PATHAK MA: Metabolic reactions in vitro of psoralens with liver and epidermis. Biochem Pharmacol 28:127–132, 1979

(*21*) PATHAK MA, MARCIANI MS, GUIOTTO A, et al: A study of the relationship between photosensitizing and therapeutic activity of 4,5′,8-trimethylpsoralen, and its major metabolite 4,8-dimethyl,5′-carboxypsoralen. J Invest Dermatol 81:533–539, 1983

Mechanisms of Photosensitization by Furocoumarins[1, 2]

Leonard I. Grossweiner[3, 4]

ABSTRACT—Furocoumarins photosensitize biomolecules to 320- to 400-nm UV by way of Type I (sensitizer-substrate) and Type II (sensitizer-oxygen) mechanisms. The Type I reactions with DNA are mediated by ground state complexes. Covalent monoadducts of the furocoumarin with pyrimidines are formed in the first photochemical step. A fraction of the monoadducts of difunctional furocoumarins are converted to interstrand cross-links in a second photochemical step, as controlled by the type of monoadduct site and the spectral distribution of the radiation. Certain furocoumarins generate singlet molecular oxygen by energy transfer from the furocoumarin triplet state. The photosensitized inactivation of enzymes involves a Type II mechanism mediated by singlet oxygen. Singlet oxygen reacts with 8-methoxypsoralen to form long-lived products, which have been implicated in the formation of covalent photoconjugates with serum albumin and other proteins and peroxidation of unsaturated lipids. The available information about furocoumarin-photosensitized inactivation of microorganisms indicates that DNA monoadducts are removed by an efficient excision repair process, and DNA cross-links are removed by a more complex, error-prone process, both of which are under genetic control. The significantly higher sensitivity of microorganisms to difunctional furocoumarins has been identified with the formation of cross-links. Both monoadducts and cross-links induce mutations in microorganisms, with a strong dependence on the specific furocoumarin structure, the strain, and the type of mutation. — Natl Cancer Inst Monogr 66: 47–54, 1984.

Photosensitization is a process in which the combined action of radiation and a sensitizing agent leads to physical, chemical, or biologic effects not observed without the sensitizer. Furocoumarins are photosensitizers of UVA, especially from 320 nm to 380 nm, a range at which cellular nucleic acids and proteins are weakly absorbing if at all. Biologic processes photosensitized by furocoumarins include enzyme inactivation, mutagenesis and inactivation of viruses, microorganisms, and mammalian cell cultures, skin erythema and tumorigenesis in laboratory animals, and the clinical end points associated with PUVA therapy. The literature on furocoumarin photophysics, photochemistry, and photobiology is voluminous. Much of this research has been done with relatively few of the many known natural and synthetic derivatives, including psoralen, angelicin, 5-MOP, 8-MOP, TMP, and 3-CP, each of which has properties relevant to either the photochemical mechanisms or clinical applications. Recent attention has been given to synthetic derivatives with high solubility in water, including 4,5′-DMA and AMT. I have attempted to review the molecular aspects of furocoumarin photosensitization, emphasizing the reactions of polynucleotides, proteins, and membrane lipids. The limited discussion of cellular systems was intended to be illustrative, and no effort toward a review of the large current literature on this aspect of the subject was made.

MOLECULAR MECHANISMS OF PHOTOSENSITIZATION BY FUROCOUMARINS

The general mechanisms by which synthetic dyes and natural pigments photosensitize chemical systems are well known. Light absorption by a sensitizer molecule populates a short-lived excited state that relaxes to the S_1 excited state within 10^{-11} seconds. A typical S_1 state releases its excess energy to lattice vibrations or by fluorescence within 10^{-8} seconds. An alternative process in essentially all photosensitizers, including furocoumarins, is the population of the metastable T_1 state by intersystem crossing from S_1. The T_1 states are more reactive than S_1 because of their longer decay lifetimes ($>10^{-6}$ seconds) and more localized electron charge distributions. It is conventional for one to subdivide T_1 reactions into two broad categories. In a Type I reaction, T_1 reacts first with a key component of the system other than molecular oxygen. In a Type II reaction, T_1 reacts first with molecular oxygen, leading to the formation of reactive intermediates. In one large class of Type II reactions, T_1 transfers its energy to unexcited (triplet) oxygen leading to the formation of singlet molecular oxygen. The $^1O_2$ state has a lifetime about 2 microseconds in water and reacts with important biomolecules, including proteins and membrane lipids. In another Type II reaction, T_1 reduces molecular oxygen to the superoxide radical anion, which is a reactive species and the precursor of hydrogen peroxide. The relative importance of Type I

ABBREVIATIONS: UVA = UV radiation at 320–400 nm; PUVA = psoralen plus UVA; 5-MOP = 5-methoxypsoralen; 8-MOP = 8-methoxypsoralen; TMP = 4,5′,8-trimethylpsoralen; 3-CP = 3-carbethoxypsoralen; 4,5′-DMA = 4,5′-dimethylangelicin; AMT = 4′-aminoethyl-4,5′,8-trimethylpsoralen; S_1 = lowest excited singlet state(s) of sensitizer; T_1 = lowest triplet state(s) of sensitizer; $^1O_2$ = singlet molecular oxygen; D_2O = deuterium oxide; C-G = cytosine-guanine; A-T = adenosine-thymine; BSA = bovine serum albumin.

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TABLE 1.—*Formation of triplet states and singlet oxygen by furocoumarins*

Derivative	Water solubility		Quantum yield							
			Triplet state				Singlet oxygen[a]			
	μg/ml	Reference	Water	Reference	Ethanol	Reference	Water	Reference	Ethanol	Reference
Psoralen	37	(*3*)	0.45	(*4*)	0.06	(*5*)	3.3	(*6*)	3.7	(*7*)
			0.12	(*5*)			7.6	(*8*)		
5-MOP	5	(*9*)	<0.01	(*4*)	0.10	(*10*)	0.52	(*6*)	4.5	(*7*)
							0.41	(*8*)		
8-MOP	23	(*9*)	0.06	(*5*)	0.04	(*5*)	1		1	
			0.14	(*4*)	0.04	(*10*)				
TMP	1	(*9*)	—		—		5.3	(*6*)	—	
AMT	10,000	(*11*)	0.20	(*12*)	0.20	(*12*)	—		—	
3-CP	13	(*9*)	0.32	(*10*)	0.46	(*10*)	26	(*8*)	—	
			>0.35	(*13*)	0.44	(*13*)				
Angelicin	20	(*14*)	0.33	(*4*)	—		≃0	(*6*)	0.9	(*7*)

[a] Each value is normalized to 8-MOP as unity.

and Type II processes in a given system depends on the local concentrations of the sensitizer, the substrates, and oxygen. Frequently used probes for $^1O_2$ include protection by $^1O_2$ acceptors and faster reaction rates in D_2O compared with H_2O, in which $^1O_2$ has a longer lifetime by a factor of approximately 20. The formation of superoxide in furocoumarin systems has not been reported.

Photophysics of Free Furocoumarins

Early work in this laboratory showed that UVA excitation of aqueous 8-MOP generates an S_1 of 1.9 nanoseconds lifetime and a longer lived T_1, which in turn produced $^1O_2$ (*1*). The production of $^1O_2$ by 8-MOP was counter to the general view that furocoumarins are "non-photodynamic" sensitizers (*2*). This finding has been confirmed for 8-MOP and extended to other furocoumarins. Table 1 summarizes data on quantum yields (species formed/photon absorbed) for T_1 and $^1O_2$ formation in aqueous and ethanolic solutions. In general, the derivatives with high T_1 quantum yields had high relative yields of $^1O_2$ formation, as expected. Detailed studies have been reported on the photophysical properties of furocoumarins, including optical absorption and emission spectra (*10, 15, 16*), intersystem crossing efficiencies and triplet–triplet absorption spectra (*1, 4, 10, 13, 16–18*), and ground state complexing of furocoumarins to polynucleotides (*9, 11, 13, 19–22*) and serum albumin (*23–25*). These data provide a basis for experimental investigations of furocoumarin photochemistry and theoretical calculations of reactivity parameters.

Primary Photochemistry of Free Furocoumarins

The flash photolysis technique has been used for measurements of T_1 reactivity with biomolecules under anaerobic conditions. The T_1 of psoralen, 3-CP, and angelicin were quenched by aqueous tryptophan and tyrosine with high rate constants, with slower reactions reported for quenching of psoralen and angelicin T_1 by histidine and phenylalanine (*4, 13*).[5] Measurements on T_1 quenching by thymine led to the surprising result that psoralen and angelicin reacted rapidly, whereas 8-MOP and TMP T_1 were at least 100-fold less reactive (*26*). A similar relationship was found for T_1 quenching of psoralen and 8-MOP by calf thymus DNA (*27*). The T_1 of psoralen and angelicin were reactive also toward uracil, cytosine, adenine, guanine, and the corresponding nucleosides (*4*). Irradiation (UVA) of furocoumarins in aqueous and organic solutions induces photobleaching and the formation of new fluorescent products. Vigny et al. (*16*) reported that 3-CP is photolyzed 100 times faster than 8-MOP and that oxygen had no effect on the reaction rates. However, Potapenko and Sukhorukov (*28*) found a significant effect of air on the photoproducts from 8-MOP in water, ethanol, and other organic solvents. Cyclobutane dimers have been identified as products of 8-MOP photolysis in dichloromethane, attributed to reactions of the T_1 (*29, 30*). Recent work has shown that $^1O_2$ reacts with 8-MOP to produce a coumarin derivative, 6-formyl-7-hydroxy-8-methoxycoumarin (*30*), the intermediates of which should be reactive with oxygen (*31*). The reaction of oxygen with the 8-MOP T_1 is indicated by indirect evidence, based on the dependence of the photoproduct yields on 8-MOP concentration (*28*). The reactions of $^1O_2$ with ground state 8-MOP or T_1 with ground state oxygen may be involved in Type II processes in which the reaction product of photolyzed 8-MOP initiates dark reactions with other substrates, such as proteins and unsaturated lipids.

Photosensitization of DNA by Furocoumarins

The pioneering work of Professor G. Rodighiero (*32*) identified the key features of DNA photosensitization by furocoumarins. It was proposed that furocoumarins form ground state complexes with DNA that are converted to covalent addition products by UVA. The reactive sites on a furocoumarin such as psoralen are the 4′,5′-double bond on the furan ring and the 3,4-double bond on the pyrone ring (sites 2–3 and 5–6, respectively, according to Ring Index nomenclature). Photosensitization of polynucleotides takes

[5] Triplet quenching refers to all reactions that induce disappearance of T_1 and includes physical quenching (deactivation) and chemical reactions.

place by C_4-cycloaddition reactions at these sites with the 5,6-double bonds of pyrimidines (*33*). Linear furocoumarins, such as psoralen, 8-MOP, and TMP, may form diadducts, which are interstrand cross-links at pyrimidine sites. The formation of cross-links requires irradiation at the spectral region of the monoadduct converted to the cross-link. For example, the 3,4-cycloadducts of psoralen are not converted to cross-links at 365 nm because of negligible absorption at this wavelength (*34*). The synthetic derivative 3-CP forms no DNA cross-links (*35*), which is related to the blocking of reaction site 3. Angelicin and its derivatives such as 4,5′-DMA form no cross-links because the molecular structure precludes the formation of 2 C_4-cycloaddition reactions at pyrimidine sites (*3, 36*). The DNA composition has a different effect on the key stages of the cross-link process. Ground state complexing of 8-methylpsoralen was approximately constant for different DNA compositions at 0.06 ligand/nucleotide and $\simeq$ 0.16 ligand/nucleotide for poly[d(C-G)]·poly[d(C-G)] and poly [d(A-T)]·poly[d(A-T)] (*19*). This result suggests that alternate sequences of purine and pyrimidine sites are most favorable for ground state complexing. The furocoumarin structure has a strong effect on ground state complexing. For example, these binding ratios for calf thymus DNA are 0.07 for 8-MOP, 0.11 for 4,5′-DMA (*19*), and unity for the highly water-soluble derivative AMT (*11*). The total photobinding of psoralen and 8-methylpsoralen increased with the A–T content of the DNA (*37*). However, these furocoumarins form 3,4- and 4′,5′-monoadducts, which do not necessarily occupy the same preferred DNA sites. Dall'Acqua et al. (*37*) deduced that 3,4-monoadducts prefer the alternate A–T sequences identified with ground state complexing, and 4′,5′-monoadducts prefer adjacent A–T and C–G sites, as maximized in DNA with 50% A–T content. The latter sites should be most favored for cross-links. Similar effects of DNA composition on total photobinding and cross-link formation were reported for 8-MOP (*38*). The total photobinding of different furocoumarins to calf thymus DNA followed the order TMP >> 4,5′-DMA > 8-MOP > 5-MOP > 3-CP (*9*). Literature data on furocoumarin photobinding to calf thymus DNA are summarized in table 2. The effect of 8-MOP binding to DNA on $^1O_2$ formation is an important question. The reactivity of $^1O_2$ with nucleic acid derivatives, proteins, and lipids is well known [e.g., the review of Foote (*40*)], and the continued generation of this damaging intermediate during the course of PUVA therapy might be significant. De Mol et al. (*41*) determined that ground state complexing of 5-MOP to calf thymus DNA increased $^1O_2$ production about threefold, and a smaller increase was obtained with 8-MOP. Evidence was obtained also for $^1O_2$ generation from psoralen after covalent photobinding. These measurements were based on the decomposition of dopa (3,4-dihydroxyphenylalanine). Recent work in this laboratory has shown that ground state complexing of 8-MOP to calf thymus DNA led to $^1O_2$ yields comparable to free 8-MOP. The assay in this case was the photosensitized inactivation of subtilisin Carlsberg (Grossweiner LI: Unpublished observations). A theoretical calculation based on in vitro parameters indicated that the quantum efficiency of $^1O_2$ encounters with the DNA surface is approximately thirty times higher than monoadduct formation when 8-MOP is complexed to DNA (*42*). The same workers demonstrated that ground state complexing is a precondition for photobinding of psoralen and 8-MOP (*42, 43*), providing quantitative confirmation of this generally accepted hypothesis.

TABLE 2.—*Covalent photobinding of furocoumarins to DNA*

Derivative	Total photobinding[a]	Reference	Cross-link formation[a]	Reference
Psoralen	1.2	(*32*)	2.0	(*3*)
5-MOP	0.63	(*32*)	0.5	(*3*)
	0.62	(*9*)	0.9	(*9*)
8-MOP	1		1	
TMP	33.1	(*9*)	14	(*3*)
			2.9	(*9*)
			2.0	(*39*)
AMT	—		4.8	(*39*)
3-CP	0.29	(*9*)	0.0	(*35*)
Angelicin	0.43	(*32*)	0.2	(*3*)
			0.0	(*36*)
4,5′-DMA	1.44	(*32*)	—	

[a]The data were obtained with calf thymus DNA and 365-nm irradiation and are normalized to 8-MOP as unity.

Photosensitization of Proteins by Furocoumarins

Subsequent to the reporting of lysozyme inactivation sensitized by 8-MOP (*1*), studies have been made with other furocoumarins and enzymes that led to complicated and not fully explained results. Prior to the investigations of enzyme inactivation, Mizuni et al. (*44*) showed that 8-MOP forms covalent photoconjugates with BSA in a relatively slow reaction, and evidence was obtained for less efficient photoconjugation of 8-MOP to several enzymes, including lysozyme, ribonuclease A, β-lactoglobulin, α-amylase, and α-chymotrypsin. Subsequent work (*24*) showed that photoconjugation of 8-MOP to BSA was faster in D_2O solutions compared with H_2O, which was indicative of $^1O_2$ involvement. However, the same 8-MOP reactivity was observed when the 8-MOP was preirradiated with UVA in oxygen and the product was mixed with BSA in the dark. This result was explained by the postulation that the 8-MOP formed a reactive product with $^1O_2$ and this product subsequently formed photoconjugates with the protein. Photoconjugation of 8-MOP was observed also with other proteins, including human serum albumin, histone, protamine, epidermal soluble protein, and the enzymes studied in the prior work (*24*). An extension of this work by Veronese et al. (*25*) showed that other furocoumarins form covalent photoconjugates with BSA, including angelicin, 4,5′-DMA, psoralen, and 8-methylpsoralen. Preirradiation photobinding was observed with 8-MOP and psoralen, and photobinding was observed for nitrogen saturation with 8-MOP, psoralen, and 4,5′-DMA. The preirradiation effect and photobinding for anaerobic conditions confirms that the reaction of $^1O_2$ with the protein is not required for photoconjugation. The indirect mechanism of Yoshikawa et al. (*24*) is supported by the observation that the extent of photomodification of the different derivatives induced by UVA without protein was

the same as the relative photoconjugation efficiency: psoralen > 8-methylpsoralen > angelicin > 4,5′-DMA > 8-MOP (*25*). Ground state complexing of 8-MOP to BSA was significant, 1.17 mol 8-MOP/mol BSA (*24*), which may facilitate photoconjugation by the anaerobic and aerobic mechanisms. The available results indicate that photosensitized inactivation of enzymes may proceed by way of several mechanisms. The involvement of $^1O_2$ was indicated in the early investigations with 8-MOP and lysozyme by the negligible rate under nitrogen and the fivefold faster rate in D_2O (*1*). Subsequent work (*45*) confirmed this result for lysozyme, but sensitized photoinactivation was not observed with 6 other enzymes with 8-MOP as the sensitizer. This was not true with other furocoumarins, especially psoralen and 3-CP which photosensitized glutamate dehydrogenase, 6-phosphogluconate dehydrogenase, and lysozyme (*46*). The role of $^1O_2$ was shown for glutamate dehydrogenase by the accelerating effect of D_2O and protection under nitrogen or in the presence of $^1O_2$ acceptors. The amino acids most reactive toward $^1O_2$ are tryptophan, histidine, methionine, and tyrosine (*40*). Photosensitized inactivation by way of $^1O_2$ should be most important for furocoumarins with high $^1O_2$ quantum yields (table 1) and for enzymes in which essential residues of these amino acids are accessible by diffusion from the external medium. Ground state complexing might promote this process when the furocoumarin binding site is adjacent to an active region of the enzyme surface. The limited data on dark binding show that ground state complexing was not important except for serum albumin (*25*). The complexity of photoinactivation is exemplified by recent research (*47*) on *Escherichia coli* DNA polymerase I in which only 2 of 3 enzymic functions were inactivated by 8-MOP in a process that required oxygen and gave positive tests for $^1O_2$. Granger and Hélène (*48*) showed that photoconjugates were formed as well; they attributed their formation to one process that does not require oxygen and a second process requiring oxygen but not $^1O_2$. The photomodified enzyme was still active, but further irradiation led to inhibition of the 5′→3′ polymerase activity and not the 5′→3′ exonuclease activity. The photoconjugation of 8-MOP to lens protein is relevant to the possible ocular damage in PUVA therapy. Experiments on human lens crystallins indicate that permanent 8-MOP photoconjugates require the presence of oxygen (*49*), a need which suggests a mechanism similar to that proposed for BSA (*24*).

Photosensitization of Membrane Lipids by Furocoumarins

The photosensitization of red blood cell hemolysis by protoporphyrin has been identified with the attack of $^1O_2$ on cholesterol (*50*). Related work showed that $^1O_2$ mediates lipid peroxidation and membrane lysis in egg phosphatidylcholine liposomes, a model membrane system (*51*). The involvement of $^1O_2$ in liposome lysis sensitized by 5-MOP and 8-MOP was first shown by Muller-Runkel (*52*) and then extended by Potapenko et al. (*53*) to indicate that the unsaturated lipids in egg phosphatidylcholine liposomes were peroxidized in the dark by the addition of 8-MOP after exposure to UVA in air. Their results suggest that $^1O_2$ reacts with 8-MOP to form a reactive product that initiates lipid peroxidation. The observation of Muller-Runkel that 8-MOP was more effective in the external aqueous medium than when incorporated in the liposomal membrane supports this conclusion. Recent experiments of Salet et al. (*54*) on photodynamic inactivation of isolated rat liver mitochondria identified an important effect of furocoumarin structure on membrane damage. Psoralen produced singlet oxygen in the respiratory medium when irradiated with UVA, which had no effect on mitochondrial functions. However, TMP impaired the respiration with rapid uncoupling of oxidative phosphorylation, as did the reference sensitizer hematoporphyrin. The differential effect was attributed to the hydrophobicity of TMP, as evaluated by the partition coefficients between 2-octanol and water, which were 800 for TMP, 470 for hematoporphyrin, and 50 for psoralen. The ineffectiveness of the putative psoralen–oxygen reaction product in this system may result from quenching by the components of the respiratory medium and the inability of the reaction product to reach the inner mitochondrial membrane.

BIOLOGIC EXPRESSION OF FUROCOUMARIN PHOTOSENSITIZATION

The photosensitization of bacterial cell cultures is the classical technique for evaluation of lethality and mutagenesis at the cellular level. A primary objective with furocoumarins has been the determination of the relative contributions of monoadducts and cross-links to these end points. In addition to the usual problems associated with survival and mutation data, including strain purity and medium dependence, the complex photochemistry of furocoumarins makes the interpretation of experimental data especially difficult. The essential photochemical factors may be summarized as follows:

1) Introduction of furocoumarin into the cell culture leads to uptake by the cells. A small fraction of the intracellular furocoumarin forms ground state complexes with the DNA for derivatives of low water solubility, such as 8-MOP. The location of the residual furocoumarin has not been determined.

2) Exposure to UVA generates covalent photoadducts from the dark-complexed fraction. For difunctional furocoumarins (such as 8-MOP), 3,4- and 4′,5′-monoadducts are formed first but not necessarily in equal numbers. As the irradiation proceeds, a fraction of the monoadducts are converted to cross-links. For example, when 8-MOP and psoralen are irradiated at 365 nm, only the 4′,5′-monoadducts are converted to cross-links.

3) Also, UVA generates $^1O_2$ from the free and complexed 8-MOP and likely from the 4′,5′-monoadducts. Possible direct targets of $^1O_2$ are membrane lipids and enzymes. The reaction of $^1O_2$ with 8-MOP leads to a long-lived reactive product that forms covalent conjugates with proteins and initiates lipid peroxidation. This process is probably a major factor in the photobleaching of the furocoumarin, which is much faster with 3-CP than with 8-MOP.

Accordingly, the biochemical lesions at an early stage of irradiation are DNA monoadducts plus a small number of cross-links and any damage to organelles initiated by the attack of $^1O_2$. Evidence is ample that the monoadducts and

cross-links are subject to repair under genetic control, e.g., the recent review of Rodighiero et al. (*55*). The available evidence indicates that monoadducts are removed by error-free excision repair, and cross-links are removed by a slower, error-prone process, the details of which are not fully explained (*56*). Comparisons of survival data for a wild-type strain with different furocoumarins provide information about the relative lethality of monoadducts and cross-links. Typical data are summarized in table 3 for 2 strains of *E. coli* and 1 strain of *Saccharomyces cerevisiae*. The order of lethality in *E. coli*, psoralen > 8-MOP > 5-MOP > angelicin > 3-CP, is the same as cross-link formation in vitro (table 2) leading to the generally accepted conclusion that cross-links are more lethal than monoadducts.

A thorough analysis of the available results by Rodighiero et al. (*55*) indicates that both unrepaired monoadducts and cross-links have comparable inherent lethalities, and the greater photosensitivity to cross-linking furocoumarins was due to the rapid and efficient repair of monoadducts. Specifically, the inhibition of scheduled DNA synthesis in Ehrlich ascites tumor cells and in mouse skin by angelicin and 4,5′-DMA was not significantly different from 8-MOP in contrast to the corresponding inactivating effects in microorganisms. A major difficulty in interpreting survival data of the type exemplified in table 3 is the lack of information about the numbers of cross-links and monoadducts at the time the cells were cultured. Furthermore, the comparison between the monoadducts of angelicin or 3-CP with the monoadducts from psoralen or 8-MOP may not be valid. An alternative approach pioneered by Cole (*60*) involves the use of repair-deficient strains. Table 4 summarizes data reported for *E. coli* K-12 photosensitization by several furocoumarin derivatives, normalized to unity for the wild-type strain for each derivative. The higher photosensitivity of the *polAl* strain to angelicin and 3-CP compared with 8-MOP indicates that DNA polymerase I is involved in the repair of monoadducts. The higher relative photosensitivity of the *uvrB* strain to 8-MOP compared with angelicin and 3-CP suggests that this gene product is involved in cross-link repair, whereas the *recA* gene product may be involved in both monoadduct and cross-link repair. I made an attempt to analyze these results in a more quantitative way by calculating the numbers of monoadducts and cross-links in the *E. coli* DNA as a function of the UVA dose, using as a basis the quantitative data for photobinding of 8-MOP to calf thymus DNA (*61*). These photoadduct yields should not depend on the repair genotype of the cell. I matched the experimental survival curves to the photoadduct yields by defining two parameters f_m and f_c, where f_m is the probability that a single monoadduct is not repaired multiplied by the probability that the unrepaired monoadduct inactivates the cell, and similarly for f_c and cross-links. The results in table 5 indicate that a cross-link is 170 times more lethal than a monoadduct in the wild-type strain, which may be attributed to the more efficient repair of monoadducts. A similar calculation for angelicin (unreported research) showed that its monoadducts had the same lethality as 8-MOP monoadducts (table 5). The lethality of monoadducts in the *polAl* strain was twentyfold higher for 8-MOP and sixtyfold higher for angelicin, supporting the role of DNA polymerase I in excision repair of monoadducts. The *recA* strain had no capacity to repair 8-MOP cross-links according to this calculation. An interesting result of the calculation is the estimate of the relative contribution of monoadducts and cross-links to inactivation at 37% survival. The

TABLE 3.—*Photoinactivation of microorganisms by furocoumarins*[a]

Derivative	Photosensitivity		
	E. coli K-12[b]	*E. coli* WP2[c]	*S. cerevisiae*[d]
Psoralen	—	3.2	—
5-MOP	—	0.57	1.6
8-MOP	1	1	1
TMP	—	—	1.7
3-CP	0.057	—	0.36
Angelicin	0.15	—	0.042
4,5′-DMA	—	—	0.25

[a] Dashes indicate data are not available.

[b] Each entry is the relative inverse dose at 37% survival for irradiation with type BL fluorescent lamps as reported in (*57*).

[c] Each entry is the relative inverse dose to 50% survival for irradiation with type BLB fluorescent lamps as reported in (*58*).

[d] Each entry is the relative inverse dose at 37% survival for 365-nm irradiation as reported in (*59*).

TABLE 4.—*Photosensitivity of repair-deficient E. coli K-12 strains to furocoumarins plus UVA*

Relevant genotype[a]	Furocoumarins		
	8-MOP	Angelicin	3-CP
Wild type	1	1	1
polAl	0.71	0.23	0.54
uvrB	0.23	0.16	0.41
recA	0.17	0.10	0.04

[a] Each entry is the relative inverse dose at 37% survival for irradiation of the *E. coli* K-12 strain with type BL fluorescent lamps as reported in (*57*).

TABLE 5.—*Calculated lethality parameters for photosensitized inactivation of repair-deficient E. coli K-12 strains*

Relevant genotype	8-MOP[a]		Angelicin	Contribution of 8-MOP monoadducts to lethality[b]
	f_m	f_c	f_m	
Wild type	0.00035	0.06	0.00035	0.47
polAl	0.007	0.06	0.0022	0.78
uvrB	0.004	0.06	0.004	0.88
recA	0.005	1.0	0.005	0.88

[a] The parameters f_m and f_c are the probabilities for inactivation by monoadducts and cross-links, respectively, multiplied by the probability the lesion was not repaired, as calculated in (*61*). The original data were reported in (*57*).

[b] Each entry is the fractional contribution of 8-MOP monoadducts to lethality at 37% survival.

calculation showed that monoadducts were more lethal in the repair-deficient strains (table 5). This result may be explained by the higher f_m values in these strains, which lead to 37% survival at lower dose rates where few cross-links are formed. A repair-deficient cancer cell may be more sensitive to monoadducts than cross-links for this reason.

The mutagenicity of furocoumarin monoadducts may be attributed to their large numbers and low lethality. Although cross-links are more lethal and fewer in number, their mutagenicity may be a consequence of error-prone repair. A recent review of furocoumarin-induced mutagenesis in bacteria and yeast cells led the authors (*55*) to conclude that both types of lesions may induce mutations, depending on the furocoumarin, the strain, and the specific mutation. A recent study on *E. coli* WP2 by Ashwood-Smith et al. (*58*) showed that the frequency of mutations $try^- \rightarrow try^+$ was psoralen > 8-MOP > 5-MOP > angelicin. The same workers found that each of these furocoumarins was a weak frameshift mutagen of the bacteria in the dark, whereas TMP was inactive in the dark. Mutation studies on haploid yeast strain N123 ($his^- \rightarrow his^+$) led to the same order for this reversion as lethality: TMP > 5-MOP > 8-MOP > 3-CP > 4,5′-DMA (*59*). However, measurements on a canavanine-sensitive clone showed that the induction of canavanine-resistant mutants was higher with angelicin than 8-MOP at 37% survival. This mutation was identified as a cytoplasmic "petite" mutation involving mitochondrial damage. The same authors found no effect of air on the lethality of *S. cerevisiae* photosensitized by angelicin, 4,5′-DMA, and 8-MOP, although there was significant protection under nitrogen with 3-CP. This result does not rule out the involvement of $^1O_2$ in view of the much higher yield with 3-CP compared with 8-MOP (table 1). Positive mechanistic tests for $^1O_2$ were reported by De Mol et al. (*62*) for mutation induction in a *uvrB* strain of *E. coli* K-12. The role of $^1O_2$ in photosensitization by furocoumarins has been the subject of much recent interest. In fact, no one has determined any good correlations of the important biologic effects in humans, psoriasis clearing, induction of erythema, and hyperpigmentation, with any simple structural property of the furocoumarin or $^1O_2$ generation in a chemical system (*55*). More information is required about the microscopic distribution of furocoumarins in epidermal cells, especially in connection with localization of the 8-MOP not dark-complexed to chromosomal DNA, if investigators are to exploit the available information about furocoumarin photosensitization of chemical systems and microorganisms.

REFERENCES

(*1*) Poppe W, Grossweiner LI: Photodynamic sensitization by 8-methoxypsoralen via the singlet oxygen mechanism. Photochem Photobiol 22:217–219, 1975

(*2*) Musajo L, Rodighiero G: Mode of photosensitizing action of furocoumarins. *In* Photophysiology (Giese AC, ed), vol VII. New York: Academic Press, 1972, pp 115–147

(*3*) Dall'Acqua F, Marciani S, Ciavatta L, et al: Formation of inter-strand cross-linkings in the photoreactions between furocoumarins and DNA. Z Naturforsch [B] 26:561–569, 1971

(*4*) Bensasson RV, Land EJ, Salet C: Triplet excited state of furocoumarins: Reaction with nucleic acid bases and amino acids. Photochem Photobiol 27:273–280, 1978

(*5*) Sa E Melo TM, Averbeck D, Bensasson RV: Some furocoumarins and analogs: Comparison of triplet properties in solution with photobiological activities in yeast. Photochem Photobiol 30:645–651, 1979

(*6*) De Mol NJ, Beijersbergen van Henegouwen GM: Relation between some photobiological properties of furocoumarins and their extent of singlet oxygen production. Photochem Photobiol 33:815–819, 1981

(*7*) Cannistraro S, Van de Vorst A: ESR and optical absorption evidence for free radical involvement in the photosensitizing action of furocoumarin derivatives and for their singlet oxygen production. Biochim Biophys Acta 476:166–177, 1977

(*8*) Vedaldi D, Dall'Acqua F, Gennaro A, et al: Photosensitized effects of furocoumarins: The possible role of singlet oxygen. Z Naturforsch [C] 38:866–869, 1983

(*9*) Dall'Acqua F, Vedaldi D, Baccichetti F, et al: Photochemotherapy of skin diseases: Comparative studies on the photochemical and photobiological properties of various mono- and bifunctional agents. Farmaco [Sci] 36:519–535, 1981

(*10*) Craw M, Bensasson RV, Ronfard-Haret JC, et al: Some photophysical properties of 3-carbethoxypsoralen, 8-methoxypsoralen and 5-methoxypsoralen triplet states. Photochem Photobiol 37:611–615, 1983

(*11*) Isaacs ST, Shen CJ, Hearst JE, et al: Synthesis and characterization of new psoralen derivatives with superior photoreactivity with DNA and RNA. Biochemistry 16: 1058–1064, 1977

(*12*) Salet C, Sa E Melo TM, Bensasson R, et al: Photophysical properties of aminoethylpsoralen in the presence and absence of DNA. Biochim Biophys Acta 607:379–383, 1980

(*13*) Ronfard-Haret JC, Averbeck D, Bensasson RV, et al: Some properties of the triplet excited state of the photosensitizing furocoumarin: 3-Carbethoxypsoralen. Photochem Photobiol 35:479–489, 1982

(*14*) Dall'Acqua F: Photochemical reactions of furocoumarins. *In* Trends in Photobiology (Hélène C, Charlier M, Monthenay-Garestier T, et al, eds). New York: Plenum Press, 1982, pp 267–277

(*15*) Pathak MA, Krämer DM, Fitzpatrick TB: Photobiology and photochemistry of furocoumarins (psoralens). In Sunlight and Man: Normal and Abnormal Responses in Man (Fitzpatrick TB, Pathak MA, Harber LC, et al, eds). Tokyo: Univ Tokyo Press, 1974, pp 335–368

(*16*) Vigny P, Gaboriau F, Duquesne M, et al: Spectroscopic properties of psoralen derivatives substituted at carbethoxy groups at the 3,4 and/or 4′,5′ reaction site. Photochem Photobiol 30:557–564, 1979

(*17*) Sloper RW, Truscott TG, Land EJ: The triplet state of 8-methoxypsoralen. Photochem Photobiol 29:1025–1029, 1979

(*18*) Bensasson RV, Chalvet O, Land EJ: Triplet, radical anion and radical cation spectra of furocoumarins. Photochem Photobiol 39:287–291, 1984

(*19*) Dall'Acqua F, Terbojevich M, Marciani S, et al: Investigation on the dark interaction between furocoumarins and DNA. Chem Biol Interact 21:103–115, 1978

(*20*) Goyal GC, Grossweiner LI: The effect of DNA binding on initial 8-methoxypsoralen photochemistry. Photochem Photobiol 29:847–850, 1979

(*21*) Tjerneld F, Norden B, Ljunggren B: Interaction between DNA and 8-methoxypsoralen studied by linear dichroism. Photochem Photobiol 29:1115–1118, 1979

(*22*) BEAUMONT PC, LAND EJ, NAVARATNAM S, et al: A pulse radiolysis study of the complexing of furocoumarins with DNA and proteins. Biochim Biophys Acta 608:182–189, 1980

(*23*) VERONESE FM, BEVILACQUA R, SCHIAVON O, et al: Drug-protein interaction: Plasma protein binding of furocoumarins. Farmaco [Sci] 34:3–12, 1979

(*24*) YOSHIKAWA K, MORI N, SAKAKIBARA S, et al: Photoconjugation of 8-methoxypsoralen with proteins. Photochem Photobiol 29:1127–1133, 1979

(*25*) VERONESE FM, SCHIAVON O, BEVILACQUA R, et al: The effect of psoralens and angelicins on proteins in the presence of UV-A irradiation. Photochem Photobiol 34:351–354, 1981

(*26*) BEAUMONT PC, PARSONS BJ, PHILLIPS GO, et al: A laser flash photolysis study of the reactivities of the triplet states of 8-methoxypsoralen and 4,5′,8-trimethylpsoralen with nucleic acid bases in solution. Biochim Biophys Acta 562:214–221, 1979

(*27*) BEAUMONT PC, PARSONS BJ, NAVARATNAM S, et al: The reactivities of furocoumarin excited states with DNA in solution. A laser flash photolysis and fluorescence study. Biochim Biophys Acta 608:259–265, 1980

(*28*) POTAPENKO AY, SUKHORUKOV VL: Luminescent photoproducts of 8-methoxypsoralen. Studia Biophys 2:15–23, 1982

(*29*) GERVAIS J, DE SCHRYVER FC: Photochemistry of some furo(3,2-g)-coumarin and 2,3-dihydrofuro(3,2-g)-coumarin derivatives. Photochem Photobiol 21:71–75, 1975

(*30*) LOGANI MK, AUSTIN WA, SHAH B, et al: Photooxidation of 8-methoxypsoralen with singlet oxygen. Photochem Photobiol 35:569–573, 1982

(*31*) WASSERMAN WH, BERDAHL DR: The photooxidation of 8-methoxypsoralen. Photochem Photobiol 35:565–567, 1982

(*32*) RODIGHIERO G, MUSAJO L, DALL'ACQUA F: Mechanism of skin photosensitization by furocoumarins. Photoreactivity of various furocoumarins with native DNA and ribosomal RNA. Biochim Biophys Acta 217:40–49, 1970

(*33*) DALL'ACQUA F, MARCIANI S, RODIGHIERO G: Photoreactivity (3655 Å) of various skin-photosensitizing furocoumarins with nucleic acids. Z Naturforsch [B] 24:307–314, 1969

(*34*) DALL'ACQUA F, VEDALDI D, RECHER M: The photoreactions between furocoumarins and various DNA with different base compositions. Photochem Photobiol 27: 33–36, 1978

(*35*) AVERBECK D, MOUSTACCHI E, BISAGNI E: Biological effects and repair of damage photoinduced by a derivative of psoralen substituted at the 3,4-reaction site. Photoreactivity of this compound and lethal effect in yeast. Biochim Biophys Acta 518:464–481, 1978

(*36*) BORDIN F, CARLASSARE F, BACCICHETTI F, et al: 4,5′-Dimethylangelicin: A new DNA-photobinding monofunctional agent. Photochem Photobiol 29:1063–1070, 1979

(*37*) DALL'ACQUA F, MARCIANI MAGNO S, ZAMBON F, et al: Kinetic analysis of the photoreaction (365 nm) between psoralen and DNA. Photochem Photobiol 29:489–495, 1979

(*38*) DALL'ACQUA F, VEDALDI D, BORDIN F, et al: Studies on the interaction between 8-methoxypsoralen and DNA in vitro. J Invest Dermatol 73:191–197, 1979

(*39*) NIELSEN PE, BOHR V: Phototoxic effect of four psoralens on L1210 cells. The correlation with DNA interstrand cross-linking. Photochem Photobiol 38:653–657, 1983

(*40*) FOOTE CS: Photosensitized oxidation and singlet oxygen: Consequences in biological systems. *In* Free Radicals in Biology (Pryor WA, ed), vol II. New York: Academic Press, 1976, pp 85–133

(*41*) DE MOL NJ, BEIJERSBERGEN VAN HENEGOUWEN GM, VAN BEELE B: Singlet oxygen formation by sensitization of furocoumarins complexed with, or bound covalently to DNA. Photochem Photobiol 34:661–666, 1981

(*42*) SHERMAN WV, GROSSWEINER LI: Photobinding of psoralen and 8-methoxypsoralen to calf thymus DNA. Photochem Photobiol 34:579–582, 1981

(*43*) GROSSWEINER LI, SHERMAN WV: The effect of dark complexing on the photosensitized formation of 8-methoxypsoralen cross-links with DNA. Photochem Photobiol 32:697–699, 1980

(*44*) MIZUNI N, TSUNEISHI S, MATSUHASHI S, et al: Some aspects on the action mechanism of 8-methoxypsoralen photosensitization. *In* Sunlight and Man: Normal and Abnormal Photobiologic Responses (Fitzpatrick TB, Pathak MA, Harber LC, et al, eds). Tokyo: Univ Tokyo Press, 1974, pp 389–409

(*45*) VERONESE FM, SCHIAVON O, BEVILACQUA R, et al: Drug-protein interaction: 8-Methoxypsoralen as photosensitizer of enzymes and amino acids. Z Naturforsch [C] 34:392–396, 1979

(*46*) VERONESE FM, SCHIAVON O, BEVILACQUA R, et al: Photoinactivation of enzymes by linear and angular furocoumarins. Photochem Photobiol 36:25–30, 1982

(*47*) GRANGER M, TOULME F, HÉLÈNE C: Photodynamic inhibition of *Escherichia coli* DNA polymerase I by 8-methoxypsoralen plus near ultraviolet radiation. Photochem Photobiol 36:175–180, 1982

(*48*) GRANGER M, HÉLÈNE C: Photoaddition of 8-methoxypsoralen to *E. coli* DNA polymerase I. Role of psoralen photoadducts in the photosensitized alterations of *pol* I enzymatic activities. Photochem Photobiol 38:563–568, 1983

(*49*) LERMAN S, MEGAW J, WILLIS I: The photoreactions of 8-methoxypsoralen with tryptophan and lens protein. Photochem Photobiol 31:235–242, 1980

(*50*) LAMOLA AA, YAMANE T, TROZZOLO AM: Cholesterol hydroperoxide formation in red blood cell membranes and photohemolysis associated with erythropoietic protoporphyria. Science 179:1131–1132, 1973

(*51*) ANDERSON SM, KRINSKY NI: Protective action of carotenoid pigments against photodynamic damage to liposomes. Photochem Photobiol 18:403–408, 1973

(*52*) MULLER-RUNKEL R: Liposome damage by oxygen intermediates and 3-carbethoxypsoralen. Ph.D. dissertation, Illinois Institute of Technology, Chicago, Ill., 1981

(*53*) POTAPENKO AY, MOSHNIN MV, KRASNOVSKY AA JR, et al: Dark oxidation of unsaturated lipids by the photooxidized 8-methoxypsoralen. Z Naturforsch [C] 37:70–74, 1982

(*54*) SALET C, MORENO G, VINZENS F: Photodynamic effects induced by furocoumarins on a membrane system. Comparison with hematoporphyrin. Photochem Photobiol 36:291–296, 1982

(*55*) RODIGHIERO G, DALL'ACQUA F, PATHAK MA: Photobiological properties of monofunctional furocoumarin derivatives. *In* Topics in Photomedicine (Smith KC, ed). New York: Plenum Press 1984, pp 319–398

(*56*) SONG P-S, TAPPLEY KJ JR: Photochemistry and photobiology of psoralens. Photochem Photobiol 29:1177–1197, 1979

(*57*) GROSSWEINER LI, SMITH KC: Sensitivity of DNA repair-deficient strains of *Escherichia coli* K-12 to various furocoumarins and near-ultraviolet radiation. Photochem Photobiol 33:317–323, 1981

(*58*) ASHWOOD-SMITH MJ, POULTON GA, BARKER M, et al: 5-Methoxypsoralen, an ingredient in several suntan preparations, has lethal, mutagenic and clastogenic properties. Nature 285:407–409, 1980

(*59*) AVERBECK D, AVERBECK S, DALL'ACQUA F: Mutagenic activity of three monofunctional and three bifunctional furocoumarins in yeast *Saccharomyces cerevisiae*. Farmaco [Sci] 36:492–505, 1981

(*60*) COLE RS: Repair of DNA containing interstrand crosslinks in *Escherichia coli*: Sequential excision and recombination. Proc Natl Acad Sci USA 70:1064–1068, 1973

(*61*) GROSSWEINER LI: Kinetics of furocoumarin photosensitization of DNA to near-ultraviolet radiation. Photochem Photobiol 34:119–123, 1981

(*62*) DE MOL NJ, BEIJERSBERGEN VAN HENEGOUWEN GM, MOHN GR, et al: On the involvement of singlet oxygen in mutation induction by 8-methoxypsoralen and UVA irradiation in *Escherichia coli* K-12. Mutat Res 82:23–30, 1981

DISCUSSION

M. Pathak: I want to know how you distinguished with 8-MOP that the original lethality was caused by a monoadduct or diadduct?

L. I. Grossweiner: We did this by analysis and computer fitting to the survival curves. Ideally, one should perform the DNA chemistry studies.

Pathak: This is the same technique used when you increase the dose of the drug; it gives you the diadduct formation.

D. Carter: Well, it means you can find cross-links after a larger dose.

Pathak: Yes.

Carter: We may be dealing with the lower limits of detection of cross-links under these clinically relevant conditions. I think that is the point.

K. Wolff: Of course, this is a negative finding. Nonetheless, with the same technique, which has its limitations, cross-links are found if higher doses are used. Can one draw conclusions as to the significance of these cross-links in the reversal of a psoriatic lesion to "normal skin?" If we use a lower dose, we cannot find them, but they may well be there.

Carter: Quantitatively, there are few. Right?

Wolff: Is this significant for treatment?

E. Riklis: When you say 6 to 12, you really mean 6–12 J/cm^2, which means 60,000–120,000 J/m^2. That is a large dose. With 6,000 J/m^2, you can have the DNA completely inhibited from replication in a monolayer cell culture. So I think the problem is not so much one of an insufficient dose. You are giving a dose, but it does not penetrate.

Pathak: That is 6 to 12 J/cm^2.

Riklis: Yes. That is 60,000 J/m^2.

Pathak: Yes, but this is precisely the therapeutic dose.

Riklis: Fine, but I am saying that the inference here is that this dose is not large enough, and that it should be increased. This is much more than you need to form cross-links in Chinese hamster cells or human fibroblasts when they are a monolayer in culture, i.e., it is not the dose because the dose you are measuring on the surface is sufficient. It is the dose at depth which is not sufficient. You probably have a mixture of cells that get no UVA and others that get a high dose.

Pathak: You and Dr. Kendric Smith are experts in this and Dr. Averbeck is here as well. We want your guidance on these studies. Can you say these are good strains to study? Can you tell us of some relationships between monofunctional and bifunctional compounds that chemists are synthesizing? We are anxious to evolve some quick assay systems. What are your recommendations?

K. Smith: You mean a definitive yes or no?

Pathak: No, sir. I want guidance. This is one of the ways we can exchange information and see whether we can improve our techniques and approaches.

Smith: Dr. Grossweiner and I were initiating an approach to this; we were trying to make use of different repair-deficient *E. coli* strains which have known defects in DNA repair. We used some compounds that were ostensibly cross-linking compounds and others that were ostensibly monoadduct formers. We investigated the survival capacity of various chemically different psoralens. Quite surprisingly, we found that the different monoformers were repaired by different mechanisms: 3-CP, 5,7-dimethoxycoumarin, and angelicin were not repaired as if they were simple monoadducts, yet the chemistry says perhaps they were. We have that kind of problem.

I would say that our paper was a beginning to try to get some sense about the different mechanisms of repair and sensitization. Certainly, all monoadduct formers are not created equal in their biologic effects.

Pathak: That is the take-home message.

K. Halprin: Dr. Pathak, is it true that cross-links have not been found in human skin treatment?

Pathak: Well, I think it is incorrect to believe that no cross-links are found. It is a dose function, dependent on the dose of both light and drug. At the dose levels that we are giving to patients, i.e., about 0.6 mg/kg and therapeutic dose levels of light of 6–12 J/m^2, we do not find cross-links.

Carter: A negative result does not exclude the presence of cross-links. However, they were not found by the technique used. Manipulation of cutaneous material was substantial before the DNA was extracted.

Chemical Basis of the Photosensitizing Activity of Angelicins[1]

Francesco Dall'Acqua, Daniela Vedaldi, Sergio Caffieri, Adriano Guiotto, Franco Bordin, *and* Giovanni Rodighiero[2]

ABSTRACT—Angelicins are a group of compounds that show marked photobiologic activity on various substrates; some of them have been proposed as potential agents for the photochemotherapy of skin diseases. A good correlation exists between the photosensitizing activity of these compounds and their capacity to induce monofunctional lesions to DNA; therefore, we believe that their photobiologic activity is essentially due to their capacity to induce photodamage to DNA. With the aim of studying the chemical nature of these photolesions, we isolated from the products of hydrolysis of the photocombinations between 5 angelicins (angelicin, 4-methyl, 5-methyl, 5′-methyl, and 5,5′-dimethylangelicin) and DNA, the corresponding new fluorescent monoadducts between the 4′,5′-double bond of the furocoumarins and the 5,6-double bond of thymine. — Natl Cancer Inst Monogr 66: 55–60, 1984.

Recently, investigators (*1–4*) have prepared and studied various methylangelicins. Their aim was to obtain new potential agents (for the photochemotherapy of psoriasis) with antiproliferative activity, due to their capacity to induce only monofunctional lesions to the skin cell DNA (*1–4*). The commonly used drugs for the photochemotherapy of skin diseases characterized by cell hyperproliferation are bifunctional furocoumarins, i.e., psoralens (*5, 6*); this treatment is beneficial, but some side effects, such as skin phototoxicity, risk of skin cancer, risk of cataracts, and possible hepatotoxicity (these last two effects may be induced only by oral administration) are present (*7, 8*).

The first two effects are related mainly to the bifunctional photolesions that psoralens induce in the skin cell DNA (*5*). Therefore, the use of the methylangelicins, which act as monofunctional compounds, should eliminate or minimize some side effects of bifunctional psoralens (*1–4*).

Although the problem of skin phototoxicity has been eliminated because methylangelicins are practically lacking in this characteristic (*1–4*), that of the risk of skin cancer is now being investigated.

Generally, the antiproliferative activity of monofunctional and bifunctional furocoumarins is due mainly to their capacity to induce photolesions to DNA. However, in regard to psoralens, various other mechanisms can be involved, such as a photodynamic mechanism by singlet oxygen formation, their photobinding to proteins, the photoinactivation of enzymes, and others (*9–12*).

Until recently, the studies performed on angelicins seemed to support the idea that, for these angular furocoumarins, the main mechanism responsible for their photosensitizing activity is connected with their capacity to photoinduce monofunctional lesions to DNA.

In fact, for numerous methylangelicins studied, good correlations between the in vitro rate constants of their photoreactions with DNA and their photosensitizing activities have been observed (*3, 13*). Bordin et al. (*1*) pointed out that these compounds photobind to DNA both in vitro and in vivo. Also, some preliminary data indicate that a correlation exists between the extent of photobinding to DNA in vitro and in vivo in a single cell system (Bordin F: Unpublished results).

Moreover, the photosensitizing activity of 4,5′-DMA on yeast was practically the same in the presence and absence of oxygen, which indicated that at least for this compound no oxygen effect is present (*14*).

The binding to the proteins and the capacity to photoinactivate enzymes (probably by way of a singlet oxygen mechanism) shown by angelicin and by 4,5′-DMA are low (*12, 15*).

Due to the biologic significance of the formation of monoadducts in DNA, we investigated the chemical nature of monofunctional lesions induced in DNA by some of these compounds.

MATERIALS AND METHODS

Furocoumarins.—Angelicin was furnished by the Franco Indian Pharmaceutical Company (Bombay, India); 4-methylangelicin, 5-methylangelicin, 5′-methylangelicin and 5,5′-dimethylangelicin were prepared by chemical synthesis in this Institute (*4*). The furocoumarins were labeled with tritiated water and catalyst by the Radiochemical Centre (Amersham, England); they were then purified by TLC [silica gel preparative plates (Merck cat. No. 5717) developed with chloroform] and had, respectively, 1, 1.01, 2.7, 4.6, and 10.4 Ci/mol.

Calf thymus DNA.—This DNA (cat. No. D1501) was purchased from Sigma Chemical Company (St. Louis, Mo.). Hypochromicity of the sample, determined according to Marmur and Doty (*16*), was over 40%.

Irradiation procedure.—Adducts, prepared from 100 ml of an aqueous solution of DNA (6.0 m*M*) containing 2 m*M* NaCl and 1 m*M* EDTA in the presence of 0.3 m*M* tritiated angelicin, were irradiated in an open cylindrical glass dish (19 cm in diameter) with 4 HPW Philips lamps emitting over 90% at 365 nm (*17*), 2 above and 2 below the dish at a distance of 7 cm for 3 hours at room temperature and under an air stream from an electric fan.

ABBREVIATIONS: 4,5′-DMA = 4,5′-dimethylangelicin; TLC = thin-layer chromatography; R_F = retardation factor.

[1] Presented at the Conference on Photobiologic, Toxicologic, and Pharmacologic Aspects of Psoralens held in Research Triangle Park, North Carolina, March 1–3, 1982.

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For the studies of the binding of tritiated psoralen (5.2 Ci/mol) to DNA and 5-methylangelicin, an aqueous DNA solution (2.3 m*M*) containing NaCl and EDTA as above and the labeled furocoumarin (3×10^{-2} m*M*) were irradiated in a system described elsewhere (*3, 13*). After irradiation, we precipitated the DNA by adding a 2 *M* NaCl solution and 2 vol of ethanol and then washed it with 80% ethanol. The DNA–furocoumarin combinations were newly dissolved in water. One part of these solutions was used for radioactivity measurements (*3*) and the remaining part was used for fluorometric determinations after acidic hydrolysis (*18*).

Hydrolysis of the DNA–angelicin combinations.—For the preparation of the adducts, to the solution mentioned above we first added solid NaCl to make a 1 *M* solution and then added 2 vol ethanol. The precipitated DNA–angelicin combination was collected with a glass rod, and, after washing with 80% ethanol and then anhydrous ethanol, it was introduced into a glass vial containing 10 ml 1 *N* hydrochloric acid; the vial was sealed and heated for 1 hour at 100° C in a boiling water bath (*19*). After cooling, the solution was neutralized with 10 *N* sodium hydroxide, added to an equal volume of ethanol, and concentrated to a small volume under reduced pressure. The concentrated solution was then chromatographed.

Photosplitting of the fluorescent photocompounds.—The filtered ethanol solutions were introduced into quartz cuvettes (1-cm optical path) and irradiated at 254 nm with a mineral light obtained from Ultra-Violet Products, Inc. (Model UVS-II; San Gabriel, Calif.) placed at a distance of 2 cm. During and after irradiation, the UV absorption spectrum of the solution was determined. After irradiation, the solution was concentrated, applied on a silica gel plate (Merck, cat. No. 5715), and developed with 9:1 ethylacetate–ethanol.

RESULTS AND DISCUSSION

Isolation of 4′,5′-Fluorescent Photoadducts

When angelicins are irradiated in the presence of DNA, a covalent combination between the 2 substances takes place (*1, 3, 4, 20*). This covalent photoconjugation is realized through the formation of monofunctional adducts between the angelicins and the pyrimidine bases of the macromolecule (*21*).

Because angelicins have 2 photoreactive sites (their 3,4- and 4′,5′-double bonds), 2 types of cycloadducts can be formed (*21*) as shown in figure 1. Based on the 3,4-monoadducts (*22*) and on 4′,5′-cycloadducts (*18*) between 4,5′-DMA and thymine recently isolated and characterized, we know that the 4′,5′-monoadducts between angelicins and pyrimidine bases of DNA are strongly fluorescent when excited with 365-nm light, whereas the corresponding 3,4-cycloadducts practically do not show fluorescence under the same conditions (*22*). Also 4′,5′-cycloadducts between psoralen and pyrimidines of DNA are strongly fluorescent when excited with UVA, but the corresponding 3,4-photoadducts are not fluorescent (*23, 24*).

We observed that, by irradiating aqueous solutions of DNA in the presence of tritiated angelicin, 4-methylangelicin, 5-methylangelicin, 5′-methylangelicin, and 5,5′-dimethylangelicin, the macromolecule acquired radioactivity and a brilliant blue fluorescence to a parallel extent (*see* fig. 2A). Therefore, the covalent combination between these furocoumarins and the macromolecule is partly due to the formation of fluorescent C_4-cycloadducts occurring between the 4′,5′-double bond of the furocoumarin and the 5,6-double bond of a pyrimidine. To isolate and characterize these fluorescent cycloadducts, we irradiated an aqueous DNA solution in the presence of the various tritiated angelicins. After irradiation, we removed the main part of unbound furocoumarin by precipitating the DNA with ethanol. The macromolecule was then hydrolyzed in an acidic medium (*19*).

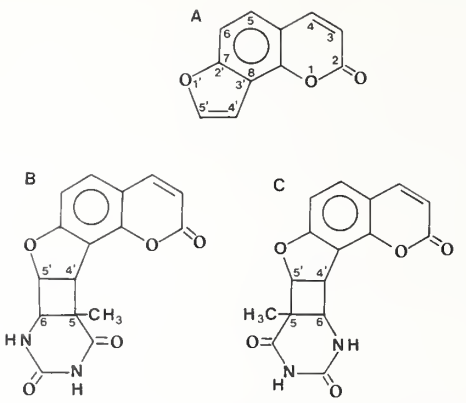

FIGURE 1.—Molecular structures of angelicin (A) and of its 4′,5′-cycloadducts with thymine (B and C).

The solution of the hydrolysis products was chromatographed on 3 MM Whatman paper developed with water–*n*-butanol–acetic acid. By this run, the fluorescent adducts (R_F between 0.7 and 0.8) were fairly well separated from the pyrimidines ($R_F = 0.6$) and intact furocoumarin (R_F around 0.9) and well separated from purines ($R_F = 0.3$). Table 1 provides the results of fluorescent bands corresponding to the adducts that were further purified on No. 1 Whatman paper developed with water from some traces of unmodified furocoumarin (R_F between 0.2 and 0.4) and thymine ($R_F = 0.5$). Two further runs were made on silica gel plates developed with 9:1 ethylacetate:ethanol (vol/vol).

The various photoadducts were eluted with ethanol, and their UV absorption spectra (fig. 3) and the excitation and fluorescence spectra were determined (table 1).

From the radioactivity of the ethanol solutions for UV absorption measurements, the concentration of the photoadducts under investigation was determined on the presumption that specific activity per mole of the starting angelicin remains unmodified after the photocycloaddition, as no loss or acquisition of hydrogen occurred in this photochemical event. The molar extinction coefficients were calculated (table 2) according to the concentration of the ethanol solutions.

Characterization of the Photoadducts

The UV absorption spectra of the various fluorescent adducts isolated are illustrated in figure 3. In figure 3B, the UV absorption spectrum of the 4′,5′-fluorescent adduct between 4,5′-DMA and thymine, recently isolated from the

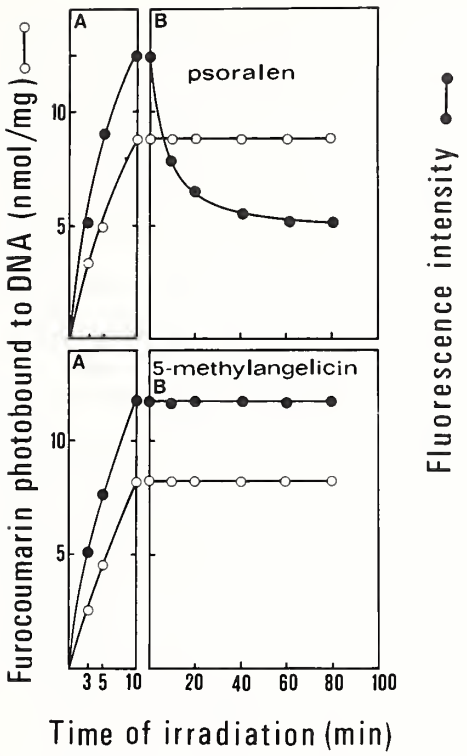

FIGURE 2.—Extents of furocoumarin covalently linked to DNA samples and the fluorescence intensities acquired by the same macromolecules irradiated in the presence of tritiated psoralen and 5-methylangelicin, respectively, are reported in "A" sections. After this irradiation (10 min), the excess of unbound furocoumarin was removed, and the macromolecules were irradiated again. In "B" sections, the behavior of the radioactivity (in amounts of furocoumarin covalently linked to DNA) and the intensity of the fluorescence due to adducts formed in the previous irradiation are reported as a function of time of irradiation.

products of hydrolysis of a DNA–4,5′-DMA combination, is shown (*18*).

We can see that the isolated photoadducts deriving from angelicin, 4-methylangelicin, and 5′-methylangelicin (fig. 3B) are almost superimposable on the above-mentioned photoadduct. Also, the adducts deriving from 5-methylangelicin and 5,5′-dimethylangelicin show strictly similar absorption spectra, even if the presence of a methyl group in the 5-position provokes a slight shift of the maximum absorption wavelength to longer wavelengths, analogous to that observed in the 2 starting angelicins (*4*).

The practical identity of the UV absorption spectra of the isolated photoadducts with that previously obtained with 4,5′-DMA is evidence that in all these new compounds, the 4′,5′-double bond initially present in the parent angelicins has been saturated (*18*). This finding is consistent with a cycloaddition at the level of this double bond. Also the activating and the fluorescence spectra (*see* table 1) of the new photocompounds are similar to those of the thymine–4,5′-DMA adduct, further supporting the identity of their chromophoric moieties.

Photodissociation of the Photoadducts

These spectroscopy data indicate that a C_4-cycloaddition occurred at the level of the 4′,5′-double bond of the various angelicins; however, they give no indication about the pyrimidine base involved in this addition.

The C_4-cycloadducts between furocoumarins and pyrimidine bases of DNA, when irradiated with 254-nm light, undergo photodissociation yielding the starting compounds (*18, 23, 24*). To ascertain which pyrimidine bases of DNA had been involved in the cycloaddition leading to the new photoadducts, we irradiated them at 254 nm in an ethanol solution.

All these compounds underwent photomodification as shown by their UV absorption spectra. After irradiation for 90 minutes, the various ethanol solutions were chromatographed on TLC. Three bands could be observed: that corresponding to the remaining initial adduct, that of thymine, and that of the starting angelicin (table 1). Again, by their UV absorption spectra, the thymine and the angelicin derivative eluted from the various plates appeared to be in equimolar amounts. Moreover, the ethanol solutions used for the UV absorption measurements were counted. Whereas thymine yielded no radioactivity, the

TABLE 1.—*Chromatographic behavior of the fluorescent photoadducts and their parent compounds*

Compound	Whatman 3MM[a]	Whatman No. 1[b]	Silica gel plate[c]	Silica gel plate[d]
Angelicin–thymine adduct	0.71	0.68	0.48	0.56
Angelicin	0.86	0.35	0.83	—[e]
5-Methylangelicin–thymine adduct	0.72	0.61	0.47	—
5-Methylangelicin	0.90	—	0.83	—
4-Methylangelicin–thymine adduct	0.78	0.64	0.42	—
4-Methylangelicin	0.86	0.33	0.84	—
5′-Methylangelicin–thymine adduct	0.68	0.80	0.68	—
5′-Methylangelicin	0.89	0.28	0.83	—
5,5′-Dimethylangelicin–thymine adduct	0.73	0.66	0.50	—
5,5′-Dimethylangelicin	0.88	0.19	0.89	—
Thymine	0.6	0.5	0.36	—

[a] Compounds were developed with the organic layer of a mixture of water–acetic acid–*n*-butanol (5/1/4; vol/vol).
[b] Compounds were developed with water.
[c] Merck (cat. No. 5715, F254); compounds were developed with ethylacetate:ethanol (9:1; vol/vol).
[d] A further purification was performed for angelicin by an additional run made by development of the plate with chloroform:ethanol (8:2; vol/vol).
[e] Dash indicates the run had not been performed for the compound.

TABLE 2.—*UV absorption capacities (in ethanol), exciting and fluorescent wavelengths of the new photoadducts*[a]

4',5'-Cycloadduct between thymine and:	Wavelengths, nm[b]												
	max	ϵ	max	ϵ	max	ϵ	max	ϵ	min	ϵ	min	ϵ	Exciting	Fluorescent
Angelicin	324	12,500	261	7,210	252	7,190	206	39,500	272	5,330	257	7,070	243	6,840	330	400
5-Methylangelicin	328	16,370	263	10,557	255	10,330	212	38,620	275	4,725	258	10,187	246	8,515	332	415
4-Methylangelicin	321	15,402	261	7,565	252	7,187	218	24,994	271	5,377	256	7,133	244	6,214	326	393
5'-Methylangelicin	323	12,468	262	5,524	252	5,500	220	22,000	271	3,356	257	5,370	246	5,198	330	395
5,5'-Dimethylangelicin	329	17,365	264	9,582	255	9,243	214	30,252	275	3,976	259	8,937	244	7,747	333	412
4,5'-DMA	322	15,490	261	6,166	251	6,166	206	38,900	271	3,715	256	5,888	244	5,623	327	390

[a] Capacities were determined in 0.05 *M* phosphate buffer, pH 6.8.
[b] max = maximum absorption; ϵ = molar extinction coefficient; min = minimum.

angelicin under investigation showed the same specific radioactivity as that of the starting compound. These data indicate that the isolated photoadducts derive from a C_4-cycloaddition between the 4',5'-double bond of the furocoumarin and the 5,6-double bond of the thymine.

Fluorometric Behavior of DNA Irradiated in the Presence of Angelicins

We observed that DNA, when irradiated in the presence of the various angelicins, acquires a brilliant blue fluorescence that increases with lengthening of the time of irradiation. From the various DNA samples so irradiated, we isolated the fluorescent monoadducts responsible for the fluorescence acquired by the macromolecule.

However, concerning the fluorescent adducts formed in the photoreactions between furocoumarins and DNA, it is well known that, whereas those deriving from bifunctional psoralens can be effectively converted into bifunctional nonfluorescent adducts [interstrand cross-linkages (*25*)], this possibility should not exist for those deriving from angelicins (*1–4, 25*). In fact, when angelicins are intercalated in duplex DNA, after the first C_4-cycloaddition involving 1 of their 2 photoreactive sites, they cannot also engage the second site (*3, 25*) because of their angular structure.

To investigate the different behavior of fluorescent monoadducts deriving from monofunctional and bifunctional furocoumarins, we irradiated a DNA solution in the presence of psoralen and of 5-methylangelicin, both of which have a similar photobinding to DNA (fig. 2A). Afterward, we removed the excess of unbound furocoumarins from the 2 samples and re-irradiated them for increasing periods.

Whereas the fluorescence of the DNA–psoralen combination decreases when irradiation time is increased (fig. 2B) because of the conversion of fluorescent monoadducts into nonfluorescent diadducts, the fluorescence of the DNA–5-methylangelicin combination remains constant, which indicates that the initially formed fluorescent monoadducts are not further modified by the second irradiation (fig. 2B). This behavior has been shown also by the other angelicins mentioned above and confirms the monofunctional character of angelicins toward native DNA and their consequent inability to induce interstrand cross-linkages in the macromolecule.

CONCLUSIONS

The fact that we easily isolated the 4',5'-fluorescent monoadducts from the products of hydrolysis of the various DNA–angelicin combinations, although we have not been able to isolate the 3,4-cycloadducts, seems to indicate that fluorescent adducts should be formed in relevant amounts in the photoreactions between angelicins and DNA.

Moreover, some preliminary data from our use of 4,5'-DMA indicate that more than 90% of its total photobinding to DNA is realized through fluorescent adducts (Dall'Acqua F, Vedaldi D, Caffieri S: Unpublished results).

Inasmuch as the photosensitizing activity of these furocoumarins appear to be due mainly to their capacity to

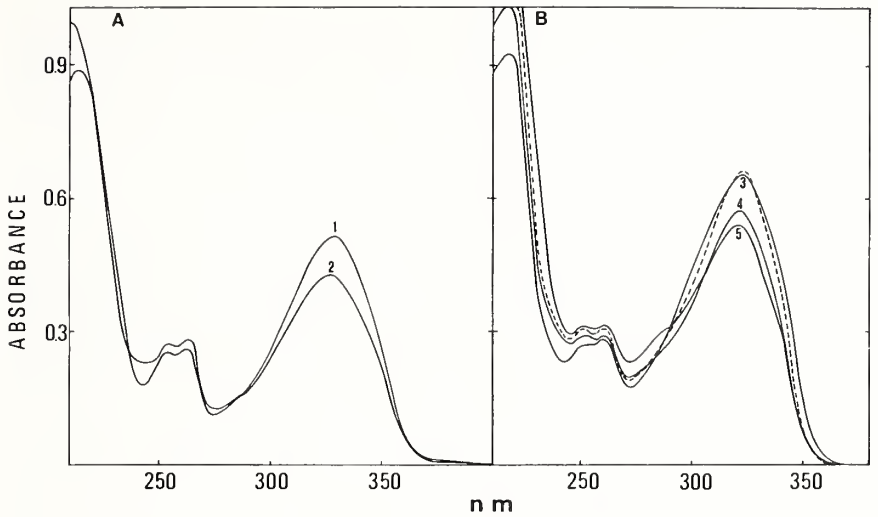

FIGURE 3.—UV absorption spectra of the isolated photoadducts between the various angelicins and thymine. A) Adducts 1 and 2 from 5,5′-dimethylangelicin (30 μM) and 5-methylangelicin (26 μM), respectively. B) Adducts 3, 4, and 5 from 5′-methylangelicin (52 μM), 4-methylangelicin (37 μM), angelicin (43 μM), respectively, and from 4,5′-DMA [(*18*); 35 μM] indicated by the *dotted line*.

induce monofunctional photolesions to DNA, 4′,5′-fluorescent cycloadducts seem to be considered the main photochemical events responsible for the photosensitizing activity of angelicins. The new fluorescent photoadducts in the DNA molecules, in contrast to those deriving from psoralen, cannot be photoconverted into nonfluorescent bifunctional adducts.

REFERENCES

(*1*) BORDIN F, CARLASSARE F, BACCICHETTI F, et al: 4,5′-Dimethylangelicin: A new DNA-photobinding monofunctional agent. Photochem Photobiol 29:1063–1070, 1979

(*2*) BACCICHETTI F, BORDIN F, CARLASSARE F, et al: Furocoumarine pour la photochimiothérapie du psoriasis et d'autres maladies de la peau qui sont lui sensibles. Belgian Patent No. 888413, August, 1980

(*3*) DALL'ACQUA F, VEDALDI D, CAFFIERI S, et al: New monofunctional reagents for DNA as possible agents for the photochemotherapy of psoriasis: Derivatives of 4,5′-dimethylangelicin. J Med Chem 24:178–184, 1981

(*4*) GUIOTTO A, RODIGHIERO P, PASTORINI G, et al: Synthesis of some photosensitizing methylangelicins, as monofunctional reagents for DNA. Eur J Med Chem 16:489–494, 1981

(*5*) PARRISH JA, FITZPATRICK TB, TANENBAUM L, et al: Photochemotherapy of psoriasis with oral methoxsalen and longwave ultraviolet light. N Engl J Med 291:1207–1211, 1974

(*6*) WOLFF K, FITZPATRICK TB, PARRISH JA, et al: Photochemotherapy for psoriasis with orally administered methoxsalen. Arch Dermatol 112:943–950, 1976

(*7*) EPSTEIN JH: Risks and benefits of the treatment of psoriasis. N Engl J Med 300:852–853, 1979

(*8*) STERN RS, THIBODEAU LA, KLEINERMAN RA, et al: Risk of cutaneous carcinoma in patients treated with oral methoxsalen photochemotherapy for psoriasis. N Engl J Med 300:809–813, 1979

(*9*) POPPE W, GROSSWEINER LI: Photodynamic sensitization by 8-methoxypsoralen via the singlet oxygen mechanism. Photochem Photobiol 22:217–219, 1975

(*10*) DE MOL NJ, BEIJERSBERGEN VAN HENEGOUVEN GM: Relation between some photobiological properties of furocoumarins and their extent of singlet oxygen production. Photochem Photobiol 33:815–819, 1981

(*11*) VERONESE FM, SCHIAVON O, BEVILACQUA R, et al: Drug–protein interaction: 8-Methoxypsoralen as photosensitizer of enzymes and amino acids. Z Naturforsch [C] 34: 392–396. 1979

(*12*) VERONESE FM, SCHIAVON O, BEVILACQUA R, et al: The effect of psoralens and angelicins on proteins in the presence of UV-A irradiation. Photochem Photobiol 34:351–354, 1981

(*13*) DALL'ACQUA F, VEDALDI D, GUIOTTO A, et al: Methylangelicins: New potential agents for the photochemotherapy of psoriasis. Structure–activity study on the dark and photochemical interactions with DNA. J Med Chem 24:806–811, 1981

(*14*) AVERBECK D, AVERBECK S, and DALL'ACQUA F: Mutagenic activity of three monofunctional and three bifunctional furocoumarins in yeast (*Saccharomyces cerevisiae*). Farmaco [Sci] 36:492–505, 1981

(*15*) VERONESE F, SCHIAVON O, BEVILACQUA R, et al: Photoinactivation of enzymes by linear and angular furocoumarins. Photochem Photobiol 36:25–30, 1982

(*16*) MARMUR J, DOTY P: Determination of base composition of deoxyribonucleic acid from its thermal denaturation temperature. J Mol Biol 5:109–118, 1970

(*17*) RODIGHIERO G, CAPPELLINA V: Ricerche sulla fotodimerizzazione di alcune furocumarine. Gazz Chim Ital (Rome) 58:551–561, 1968

(*18*) DALL'ACQUA F, CAFFIERI S, VEDALDI D, et al: Monofunctional 4′,5′-photocycloadduct between 4,5′-dimethylangelicin and thymine. Photochem Photobiol 33:261–264, 1981

(*19*) DALL'ACQUA F, CAFFIERI S, RODIGHIERO G: Fluorimetric determinations of 4′,5′-cycloadducts in the DNA–psoralen photoreaction. Photochem Photobiol 27:77–79, 1978

(*20*) RODIGHIERO G, MUSAJO L, DALL'ACQUA F, et al: A comparison between the photoreactivities of some furocoumarins with native DNA and their skin-photosensitizing activity. Experientia 25:479–481, 1969

(*21*) DALL'ACQUA F: Photochemical reactions of furocoumarins. *In* Trends in Photobiology (Hélène C, Charlier M, Monthenay–Garestier TH, et al, eds). New York: Plenum Press, 1981, pp 267–278

(*22*) DALL'ACQUA F, CAFFIERI S, VEDALDI D, et al: Monofunctional 3,4- and 4′,5′-photocycloadducts between 4,5′-dimethylangelicin and thymine. Photochem Photobiol 37:373–379, 1983

(*23*) MUSAJO L, BORDIN F, CAPORALE G, et al: Photoreactions

at 3,655 Å between pyrimidine bases and skin-photosensitizing furocoumarins. Photochem Photobiol 6:711–719, 1967

(*24*) MUSAJO L, BORDIN F, BEVILACQUA R: Photoreactions at 3655 Å linking the 3,4-double bond of furocoumarins with pyrimidine bases. Photochem Photobiol 6:927–931, 1967

(*25*) DALL'ACQUA F, MARCIANI S, CIAVATTA L, et al: Formation of interstrand cross-linkings in the photoreactions between furocoumarins and DNA. Z Naturforsch [B] 26:561–569, 1971

SESSION III

Pharmacologic and Metabolic Aspects of Psoralens and Longwave Radiation Therapy

Pharmacokinetics and Pharmacodynamics of Psoralens After Oral Administration: Considerations and Conclusions[1]

Rolf Brickl, Jochen Schmid, *and* Friedrich W. Koss[2, 3]

ABSTRACT—We discovered a strong but saturable first-pass effect after oral administration of psoralens by using different doses and simultaneous or timed application of stable isotopes. Therefore, small variations of dose, disintegration of drug, and amount and rate of absorption gave rise to great differences in plasma levels and therapeutic efficacy. For practical therapy, the following conclusions can be drawn: 1) Galenical forms of psoralens should ensure a quick and highly reproducible absorption. 2) In the event that inefficacy has been detected, plasma levels should be determined, and the psoralen dosage should be increased rather than the irradiation doses in most instances. 3) For oral psoralen and 320- to 400-nm UV (UVA) treatment, a combination of 5-methoxypsoralen (5-MOP) and 8-MOP (with a lower dose and either administered 30 minutes later than the 5-MOP or in a drug product with quick release of 5-MOP and quick but delayed release of 8-MOP) results in much higher efficacy and reproducibility. Therefore, compared with the single drug, in the combination, dose of drug and the amount of irradiation can be reduced considerably which may result in increased safety. 4) Plasma levels after oral administration of dissolved 4,5′,8-trimethylpsoralen are low, but phototoxicity is comparable to that of the 5-MOP and 8-MOP. — Natl Cancer Inst Monogr 66: 63–67, 1984.

Even though oral PUVA treatment has become popular during the last decade and is used worldwide, most of the improvements in therapy are based more on practical experience. Little progress stems from basic research on this complicated treatment of skin diseases. Because of the risk of long-term hazards, an optimized therapy in regard to standardization of treatment regimen, reduction of side effects, drug dosage, and amount of irradiation is necessary (*1–8*). Here we present both a rational approach based on theoretical considerations and on in vitro and in vivo experiments and our conclusions for therapeutic use.

MATERIALS AND METHODS

The 8-MOP-d_3 was synthesized from 8-hydroxypsoralen and trideutero dimethyl sulfate. Both 8-and 5-MOP (purity >99%) were administered to 3 male healthy volunteers as solutions in ethanol:solketal (1:1) to avoid the variations caused by differences in disintegration time of tablets.

At the different sampling times blood was drawn from the anticubital vein into heparinized syringes, and plasma, obtained by centrifugation, was frozen until analysis. Determination of plasma levels was done by gas chromatography or high-performance liquid chromatography (lower limit of detection was 5 ng/ml). We used a Kromayer high-pressure mercury lamp Q (121 U) from Hanau (Federal Republic of Germany). Using a Schott filter (WG 345, 2 mm), we cut off the UV radiation at 290–320 nm; light intensity at 366 nm was 22 milliwatts/cm^2. A special mask placed on irradiation sites of patients permitted simultaneous irradiation of 6 patches of skin, each with a diameter of 7 mm. The degree of erythema and pigmentation reactions were assessed on an arbitrary scale of 0 to ++++.

RESULTS AND DISCUSSION

The efficacy of oral PUVA treatment depends on 3 facts: 1) amount of psoralen at the site of photochemical action in the skin, 2) amount of radiation and wavelength of light which reaches the site of action in the skin, and 3) intrinsic activity of the combination of psoralen plus irradiation.

The important steps between the ingestion of a psoralen and its arrival at the site of action that are relevant for all kinds of drugs include disintegration and dissolution of drug, absorption, first-pass effect, concentration in central compartment (plasma), tissue distribution, and concentration at the site of action.

In figure 1, a strong nonlinearity is evident that indicates a saturable first-pass effect. Therefore, some of the factors influencing the availability of the drug at the site of action mentioned above are even more important when PUVA treatment is administered than with other drugs.

Figure 2 shows that before reaching the central compartment blood, a drug can be metabolized either during the passage through the gut wall or in the liver by way of the portal vein. For 8- and 5-MOP, the primary site of biotransformation is oxidation of the furan moiety. Therefore, metabolism of the MOP in the liver is more likely than biotransformation in the intestine or during absorption. The proof for such a saturable first-pass effect is given in figure 3; the second dose produces much higher plasma levels.

In an independent experiment, a possible isotopic effect was excluded; when 8-MOP and 8-MOP-d_3 were given simultaneously, both drugs showed identical pharmacokinetic behavior.

If the area under the curve is plotted versus the dose (fig. 4), a threshold dose can be estimated. This suggests

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ABBREVIATIONS: PUVA = psoralen plus UV radiation at 320–400 nm; 8-MOP-d_3 = trideuterated form of 8-methoxypsoralen; 5-MOP = 5-methoxypsoralen; TMP = 4′,5′,8-trimethylpsoralen.

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[2] Department of Biochemistry, Dr. Karl Thomae GmbH, Chemisch-Pharmazeutische Fabrik, Postfach 1755, 7950 Biberach 1, Federal Republic of Germany.

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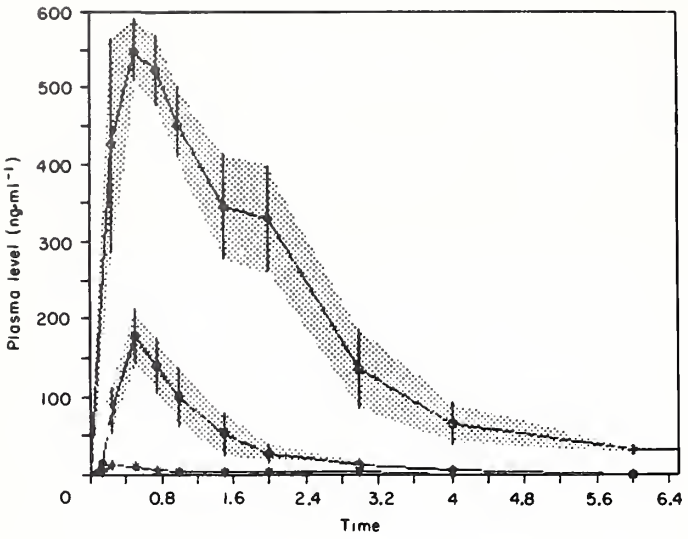

FIGURE 1.—Plasma levels of 8-MOP administered orally as a solution of different doses: * = 10 mg; ● = 20 mg; O (top) = 40 mg. Time is in hours.

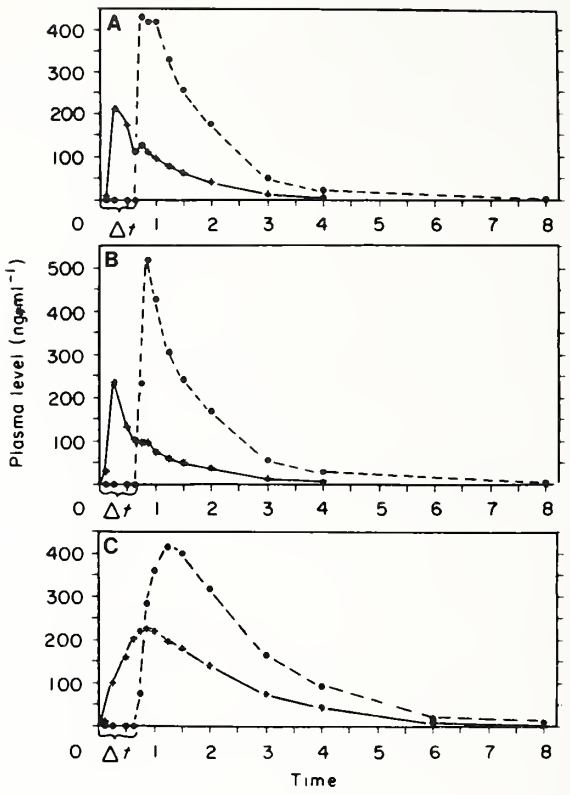

FIGURE 3.—Plasma levels of 20 mg 8-MOP (*) and 20 mg 8-MOP-d_3 (●) given 37.5 min later, both in solution, to 3 subjects: RG (A), EHS (B), and FB (C). Time is in hours.

that doses below this threshold dose are completely metabolized and do not produce any detectable plasma levels.

Among the practical consequences for therapy of this saturable first-pass effect, we find that the metabolizing capacity of the liver is limited, and the amount of drug that can be metabolized and therefore is therapeutically inactive depends on the influx rate of MOP in the liver, which means that the first-pass effect depends on the absorption rate and dose of MOP.

The absorption rate depends on the amount of drug administered and the rate of dissolution (fig. 5). Therefore, small differences in these parameters give rise to large differences in plasma levels.

Figure 6 illustrates some theoretical curves simulated by a computer program. Disposition rate constants of MOP were experimentally determined, and absorption rates were arbitrarily chosen. It is evident that, with constant dissolution and absorption rates, the amount of administered drug plays an important role because of the strong nonlinearity. At a constant level of drug, the rate of dissolution, i.e., the

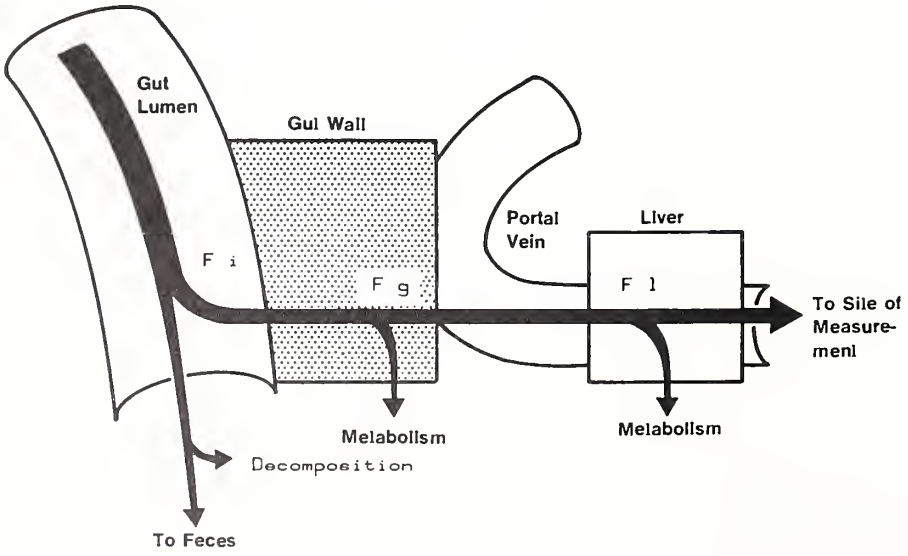

FIGURE 2.—Possibilities for loss of drug by first-pass effect. F_i, F_g, F_l = fractions entering the organs.

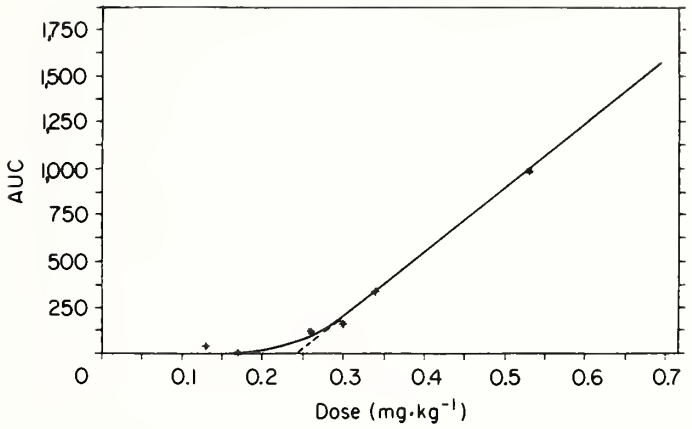

FIGURE 4.—Plot of dose of 8-MOP vs. area under the curve (AUC) of plasma level curve.

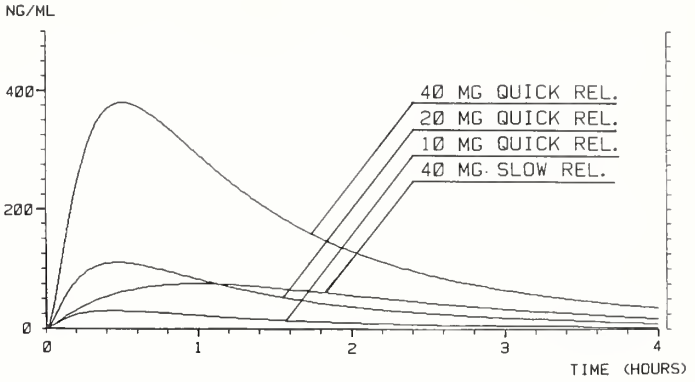

FIGURE 6.—Calculated plasma levels at different doses and different rates of dissolution. Rel. = release.

galenical properties of the drug, are most important. This special pharmacokinetic behavior may explain the large differences in plasma levels that occur when different brands of psoralen tablets are administered. Food intake may also have a strong influence. An example of the great importance of release characteristics of the drug product is given in figure 7.

Although oral administration of 40 mg TMP in solution results in extremely low plasma levels, the drug shows a strong phototoxic reaction comparable to MOP. Kligman (*5*) reported that even when TMP is given orally in a high dose of 200 mg, it is inactive (nonphotosensitizing). However, a correlation between plasma levels and phototoxic reactions has been demonstrated. That there must be some relationship between plasma levels and therapeutic activity is clear because the drug can reach the site of action only through the blood. A plasma level at any given time is the result of absorption rate, first-pass effect, distribution in the body, and elimination and metabolism of the drug.

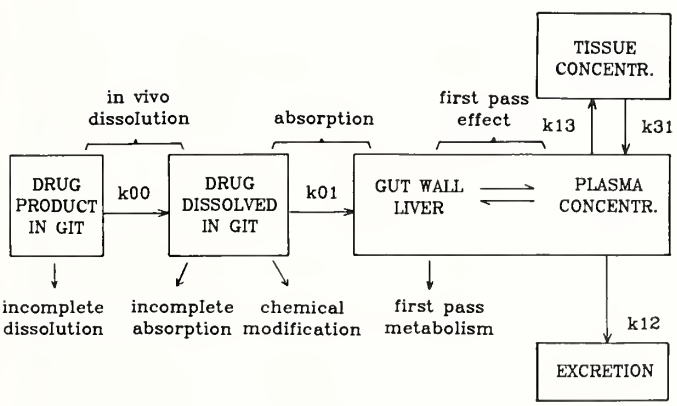

FIGURE 5.—Model for pharmacokinetics of psoralens. GIT = gastrointestinal tract; concentr = concentration. Absorption kinetics: k00 = rate constant of dissolution; k01 = constant of absorption. Disposition kinetics: k13 = rate constant of diffusion into side compartment; k31 = rate constant of back diffusion from side compartment; and k12 = rate constant of excretion.

Because the therapeutic activity depends on the amount of drug in the skin, it seems reasonable for one to assume that this is not a simple and mathematically exact correlation. The results shown in figure 7 support this assumption. The intrinsic activity of TMP is only about three to four times greater than that of MOP, whereas the plasma level was

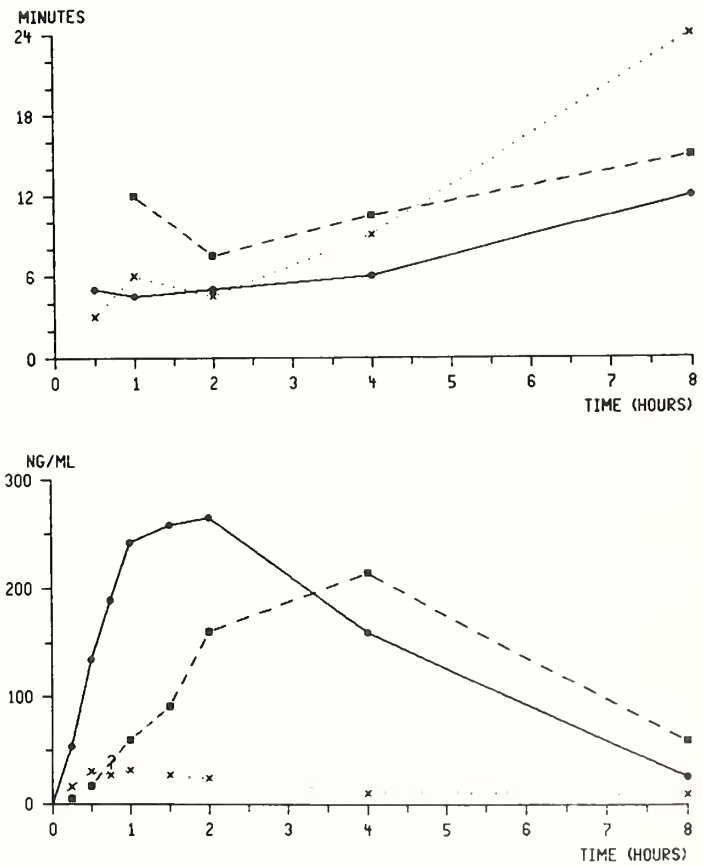

FIGURE 7.—Comparison of plasma levels (*upper*) and phototoxicity (*lower*) of different psoralens after 40 mg 8-MOP (●), 5-MOP (■), or TMP (X) were given in solution to subject SB.

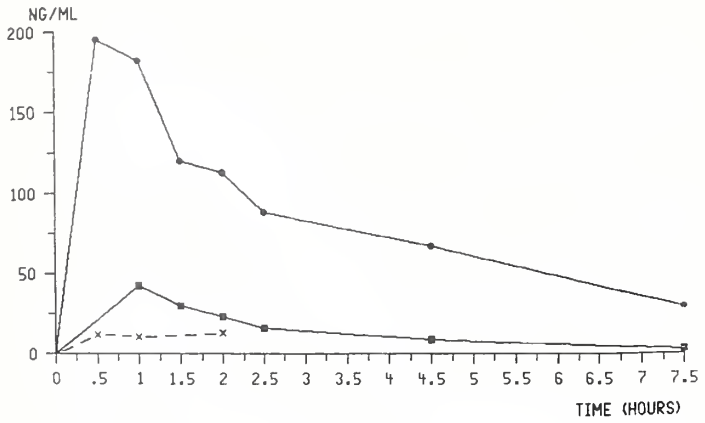

FIGURE 8.—Plasma levels of single administration of 10 mg 8-MOP (-----x-----), after combined administration of 40 mg 5-MOP (——●——), and 10 mg 8-MOP 30 min later (——■——).

only about 5% that of MOP. However, the phototoxic activity was similar. If one looks at the time course of plasma levels and the phototoxic reaction, a similar profile can be proved, but the phototoxic response has a greater lag time than does the plasma level, and the phototoxic reactivity decreases much more slowly than does the plasma level. At times when plasma levels of the parent compound approach zero, the phototoxic reaction is still high. This fact is also therapeutically important because it proves that protection of the skin must last at least 24 hours.

Knowledge of pharmacokinetics and pharmacodynamics of drugs and the consequent application of this knowledge may result in improved dosage regimens. The most common drug for oral PUVA treatment is 8-MOP. Recently, Hönigsmann et al. (*2*) also used 5-MOP. The main differences between the 2 drugs are: 1) The 8-MOP is more phototoxic than is 5-MOP and shows a relatively smaller therapeutic range between ineffective and strongly erythemogenic light doses. The amount of drug given orally is limited by gastrointestinal side effects, such as nausea. 2) The 5-MOP is less phototoxic than is 8-MOP, and, therefore, the dose of light shows a wide therapeutic range. Neither side effects of the gastrointestinal tract nor those of the skin, such as erythema, pruritis, or blistering, occur at higher doses in contrast to those resulting from therapy with 8-MOP. 3) If 5-MOP is given in a dose of 1.2–1.6 mg/kg (twice the dose of 8-MOP), length and frequency of treatment required are about one-third less than with 8-MOP, but the necessary UVA dose is the same for both treatments. 4) Erythema with 5-MOP peaks at about 48 hours, then it subsides rapidly, and then the pigmentation response becomes visible, whereas the 8-MOP-induced erythema response peaks at 96 hours and gradually turns into increasing pigment response.

From the pharmacokinetics of psoralens (saturable first-pass effect) and the differences in pharmacodynamics, the following conclusions for a rational, highly effective, and reliable therapy can be derived:

The first-pass effect, which can cause great variations in plasma levels and therefore also in reactivity to light, should be saturated with 5-MOP because it allows high doses of drug and light without the potential risk of severe burning.

If the first-pass effect is saturated, a low dose of 8-MOP, which does not produce gastrointestinal side effects, gives high and reproducible plasma levels. Therefore, an exact dosing of light is possible that yields the required but barely visible erythema without the danger of severe burning.

Because erythema induced by both compounds peaks at different times, a single dose of irradiation should give a long-lasting effect if both compounds are dosed in a ratio which matches the differences in phototoxicity. Therefore, therapy with a reduced number and frequency should be possible.

The experimental proof for these conclusions is given in figure 8 and table 1, which show plasma levels and sensitivity to UVA after a single administration of 10 mg 8-MOP and the combined administration of 40 mg 5-MOP and 10 mg 8-MOP 30 minutes later. Administration of a 10-mg dose of 8-MOP (which is less than the calculated

TABLE 1.—*Erythema readings after 72 hr*[a]

Irradiation after: hr	Psoralen	UVA dose, J/cm²						
		1	2	4	6	9	12	18
1	8-MOP	—	0	0	0	0	0	+
	5-MOP	0	0	0	(+)	(+)	+	—
	Combination	+	+	+++	++++	++++	++++	—
2.5	8-MOP	–	0	0	0	0	(+)	(+)
	5-MOP	0	0	(+)	(+)	+(+)	++	—
	Combination	(+)	+	+(+)	++(+)	+++	++++	—
4.5	8-MOP	—	—	—	—	—	—	—
	5-MOP	—	0	(+)	(+)	(+)	+(+)	+(+)
	Combination	0	(+)	(+)	(+)	+(+)	++(+)	—
8	8-MOP	—	—	—	—	—	—	—
	5-MOP	—	0	0	(+)	(+)	(+)	+(+)
	Combination	—	0	(+)	+	+	+	+(+)

[a] Dashes indicate readings were not done. Plus signs *in parentheses* indicate a weak reaction.

threshold dose of ~14 mg or 0.24 mg/kg body weight) produces low plasma levels, whereas the same dose after pretreatment with 40 mg 5-MOP results in plasma levels that are about four times as high. This combination also increases the sensitivity to UVA (table 1).

Table 1 shows the combination treatment to be about three times more erythematous than 5-MOP alone, but the 8-MOP in this low a dosage is nearly ineffective.

The difference is greater 1 and 2 hours after dosing, i.e., at the times of maximum sensitivity. Later on, with the low plasma levels, the first-pass effect is no longer saturated, and, therefore, the potentiating effect of the combination is diminished. High sensitivity during this period would be a drawback, however, because it might result in overdosing of UVA and considerable erythema.

This demonstrates that the proper combination therapy with both drugs provides the advantages of both and should allow PUVA therapy with lower doses of the single drugs, markedly lower doses of radiation for a single treatment, and a reduced number of treatments. Because the possible risks of PUVA therapy are almost certainly related to the amount of radiation which has to be given, safer therapy may result.

REFERENCES

(1) Goldstein FP, Carter DM, Ljunggren B, et al: Minimal erythema doses and psoralen plasma levels in PUVA patients. Clin Res 28:568, 1980

(2) Hönigsmann H, Jaschke E, Gschnait F, et al: 5-Methoxypsoralen (bergapten) in photochemotherapy of psoriasis. Br J Dermatol 101:369–378, 1979

(3) Swanbeck G, Ehrsson H, Ehrnebo M, et al: Serum concentration and phototoxic effect of methoxsalen in patients with psoriasis. Clin Pharmacol Ther 25:478–480, 1979

(4) Ehrsson H, Nilsson SO, Ehrnebo M, et al: Effect of food on kinetics of 8-methoxsalen. Clin Pharmacol Ther 25:167–171, 1979

(5) Kligman AM, Goldstein FP: Ineffectiveness of trioxsalen as an oral photosensitizer. Arch Dermatol 107:413–414, 1973

(6) Schmid J, Prox A, Reuter A, et al: The metabolism of 8-methoxypsoralen in man. Eur J Drug Metab Pharmacokinet 5:81–92, 1980

(7) Schmid J, Prox A, Zipp H, et al: The use of stable isotopes to prove the saturable first-pass effect of methoxsalen. Biomed Mass Spectrum 7:560–564, 1980

(8) Stolk L, Kammeyer A, Cormane KH, et al: Serum levels of 8-methoxypsoralen; difference between two oral methods of administration. Br J Dermatol 103:417–420, 1980

Quantitative Tests for Psoralens in the Blood: Methods and Uses in Monitoring Psoralen and Longwave Radiation Therapy[1]

D. Martin Carter and David P. Goldstein[2]

ABSTRACT—Variability in 2 factors determines the therapeutic success of photochemotherapy with psoralens and UV radiation at 320–400 nm (PUVA): biochemical properties of the photosensitizing drug used and characteristics of the source of irradiation. Fine-tuning of PUVA therapy is achieved by delivery to the patient of the phototoxic dose of UV radiation at the precise time of peak bioavailability of the photosensitizing psoralen; thus it depends on accurate means for measuring the 2 variables. The UV emission is easily measured, but bioavailability of photosensitizing drugs is not. Blood levels provide information about the photosensitivity of the patient and thus relate to therapeutic efficacy. The oral dose of 8-methoxypsoralen (8-MOP) cannot be used to predict what plasma levels of the drug will be achieved. Dissolution and absorption of 8-MOP are affected by the form of the preparation. Absorption rates vary far more than do elimination rates in the same population of patients, but individual differences in metabolism of the drug have not been documented. Maximum therapeutic benefit varies from individual to individual. It may be influenced by factors affecting absorption, distribution, and metabolism of the drug and by skin type and degree of skin pigmentation. — Natl Cancer Inst Monogr 66: 69–72, 1984.

Variability in 2 factors determines the therapeutic success of photochemotherapy with psoralens and UVA: biochemical properties of the photosensitizing drug used and characteristics of the source of irradiation. A reasonable goal for photochemotherapists is for them to realize maximum therapeutic benefit from PUVA therapy and to reduce the acknowledged risks to a minimum. The patient can best achieve maximum therapeutic benefit by receiving the phototoxic dose of UV irradiation at the precise time of peak bioavailability of the photosensitizing psoralen. Fine-tuning of PUVA therapy thus depends on accurate means for measurement of the 2 therapeutic variables.

Sophisticated technology exists for producing efficient UV lamps and radiometers for measuring their emission. Scientists' attempts to quantitate the bioavailability of photosensitizing drugs have been less successful. Photo patch-testing is one excellent method for one to determine the time of a patient's maximum photosensitivity, but it requires that subjects be repeatedly evaluated for several days. Quantitative measurements of psoralen levels in the skin are being tried, but they have not been practical (*1*). However, measurements of psoralen blood levels have been attempted by several investigators, and the techniques are becoming simplified. The assumption has been that blood levels provide information about the photosensitivity of the patient and thus relate to therapeutic efficacy.

Each of the methods developed for determination of psoralen concentration in plasma or serum requires that the drug be extracted in an organic solvent. Various systems have been used for separation and resolution of the extract for detection of psoralen. In most, the investigators have been seeking to measure the concentration of 8-MOP, but similar methods can be used for other psoralens. Thin-layer chromatography and scanning fluorometry (*2–4*), gas–liquid chromatography, flame ionization detection (*5*), electron capture detection (*6, 7*), HPLC, and UV absorption spectrometry (*8–11*) have been used. One sensitive and complex system involves HPLC followed by glass capillary gas–liquid chromatography and mass spectrometry detection (*12*). Many of these methods are unsuitable for rapid and repeated determinations of 8-MOP plasma profiles because they are time-consuming and expensive, require excessive sample size, or lack sensitivity. We have used a rapid and inexpensive HPLC system which can measure 8-MOP plasma concentrations of 4 ng/ml from 1 ml plasma (*13, 14*).

In our procedure, 8-MOP is extracted from plasma in benzene (*10*). The concentration of 8-MOP is determined by UV absorbance after separation by HPLC in a silica-particle column eluted with methylene chloride:acetonitrile (95:5). The simple, one-step extraction of 8-MOP with benzene that we used is sufficient for excellent recovery of the psoralen. Multiple organic-phase extractions (*4, 5*), acidification for release of psoralen from plasma proteins (*4, 7*), or other complex clean-up procedures (*6*) are not necessary. Although thin-layer chromatography systems with fluorodensitometric determination are sensitive to within 5 ng/ml with as little as 1 ml serum (*3*), all such systems (*2–4*) lack specificity for detection of 8-MOP alone; purification steps are required for gas chromatography systems (*5–7*). The sensitivity of the HPLC method we have used is comparable to that of others (*9–11*). Multiple samples of venous blood can be obtained from a single patient because only 1 ml plasma is required for each determination.

We found it necessary to treat all glassware with polyethylene glycol to prevent nonspecific binding of 8-MOP to glassware. Ammidin was added as an internal standard before the extraction; thus we could recognize variations in extraction efficiency in different samples.

ABBREVIATIONS: UVA = UV radiation at 320–400 nm; PUVA = psoralen plus UVA; 8-MOP = 8-methoxypsoralen; HPLC = high-pressure liquid chromatography; MPD = minimum phototoxic dose(s).

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Ammidin is an isoamylene derivative of 8-oxypsoralen with UV absorption characteristics similar to those of 8-MOP, but it has a more rapid elution time in this HPLC system. It is an ideal internal standard. Plasma extraction of 8-MOP is slightly more efficient than is serum extraction with our technique (*13*).

In our clinical studies of PUVA-treated patients, we found individual variability in 8-MOP plasma profiles, peak 8-MOP levels, and time of peak level (*14*). This could not be explained by patient differences in oral dose of drug. The recommended oral dose of 8-MOP in the protocol for the cooperative clinical trials in the United States is 0.50 to 0.70 mg/kg body mass (*15*). Most of our patients received this dosage schedule. In early studies, we determined that ingestion of 40–60 mg 8-MOP was required to produce perceptible erythema, but 80- to 100-mg oral doses resulted in edema and blistering (*16*). Steiner and his group (*2*) reported no correlation in 37 patients between the 8-MOP serum levels at 2 hours and the oral dose or the response to irradiation. Wagner and colleagues (*17*) performed complete 8-MOP profiles on 21 patients ingesting comparable amounts of drug and could not account for variations in peak 8-MOP levels or measurements of the area under the curve. The oral dose of 8-MOP cannot be used to predict what plasma levels of the drug will be achieved.

We observed that elimination rates of the parent molecule obey first-order kinetics (*14*). Elimination half-lives ranging from 1.1 to 1.9 hours have been reported on small groups of patients (*11, 18, 19*). We found no effect from the ingestion of food on the elimination rate of 8-MOP, which is in agreement with Ehrsson et al. (*19*). In studies of radiolabeled 8-MOP elimination, Busch and co-workers (*20*) found that the decline of radioactivity was first order and biphasic, with a rapid initial half-life and a terminal half-life of about 200 hours. Small quantities ($<1\%$) of 8-MOP metabolites may linger much longer than the parent molecule (*18, 20*). Radiolabeled 8-MOP has been recovered in high concentrations in liver, kidney, and adrenals of rats soon after oral or iv administration (*21*). Psoralens are extensively transformed to polar metabolites by hydroxylation and glucuronidation (*20, 22, 23*). In human beings, 80% of orally administered 8-MOP is excreted in the urine within 8 hours (*22*), but lesser quantities of polar metabolites are eliminated in the urine and feces over several days (*20*). After absorption, 8-MOP is rapidly metabolized with consistent elimination rates in the population.

Absorption patterns show great variation in the population. We have seen that the time-course patterns for 2 individuals may be markedly different, even though oral doses and elimination rates are similar (*14*). Other investigators have also observed wide variations of absorption patterns (*3, 11, 13, 17–19, 24, 25*).

Food intake may account for some of this variation. Five of our patients and subjects in other studies demonstrated biphasic absorption patterns (*14, 20, 25*). When food intake was restricted in 2 of our patients, the absorption patterns became monophasic, with earlier and greater peak levels of the drug. Some of our patients repeatedly demonstrated similar absorption patterns under fasting conditions. Gazith et al. (*18*) reported that fasting subjects had greater overall 8-MOP levels than did those who were nonfasting but ingesting the same amount of drug. Ehrsson and associates (*19*) reported that food ingestion enhanced absorption of the drug in 5 subjects. In practice, clinicians often make specific recommendations regarding food intake with 8-MOP administration to counter the nausea that some patients experience after ingesting the drug. A consistent and reproducible plasma profile is desirable so that a favorable therapeutic response to a treatment plan is ensured. We now advise patients to minimize food intake and maintain consistent dietary habits during PUVA treatments.

Individual differences in the ability to dissolve the orally administered drug formulations may account for some variation in absorption patterns. Thune and Volden (*9*) suggested that the dissolution rate may affect the plasma level of the drug. Absorption rate constants have a much greater variation than do elimination rate constants in the same population of patients (*19*). Food content in the upper gastrointestinal tract is likely to affect dissolution rates. Several studies have shown that liquid preparations of 8-MOP have greater and more rapid absorption than do crystalline preparations (*7, 25, 26*). Radiolabeled 8-MOP was administered orally to human subjects in equivalent doses in 2 forms: a coarse crystalline preparation and a liquid preparation (*20*). Seventy percent of ingested 8-MOP in the liquid form was absorbed, but only 30% in the crystalline preparation, as measured by urinary elimination. The fecal radioactive content was predominantly in polar metabolites of 8-MOP. This finding may be explained by dissolution and opening of the lactone ring of 8-MOP, as occurs in alkaline conditions like those existing in the small intestine (*6, 7*). Variable gastrointestinal transit times also account for the variation in absorption patterns that were seen in this and other studies. We (*13*) and others (*27*) have shown variations in the bioavailability of different manufacturers' crystalline preparations. Investigators in several countries using different 8-MOP crystalline preparations report wide variations in 8-MOP plasma concentrations that are partly explained by differences in available drug formulations (*2–11*). We found no variability in the quantitative chemical content of commercial 8-MOP capsules as they are provided by several manufacturers (*13*). It is not customary for scientists to compare the bioavailability of different batches of 8-MOP as provided by the same or different manufacturers. A preparation of 8-MOP that ensures rapid and consistent dissolution and absorption would be most desirable.

The relative rates of drug metabolism and distribution are also presumed to contribute to interindividual variations in 8-MOP absorption patterns. Appreciable concentrations of 8-MOP and its metabolites have been identified in plasma within 15 minutes of ingestion (*20*). Both metabolites and the parent molecule followed parallel absorption and elimination patterns (*18, 20*). This suggested a first-pass phenomenon for 8-MOP metabolism. Greater bioavailability of the liquid compared with solid formulations may be explained by greater first-pass metabolic degradation in the liver of the solid preparations of the drug caused by slow capsule dissolution in the gastrointestinal tract (*25*). No studies have been published

that document individual differences in metabolism of the drug. In laboratory animals, 8-MOP is preferentially distributed to liver, kidney, adrenal, skin, and blood, in that order (*21, 22*). Wulf and Hart (*21*) determined that the concentration of 8-MOP in skin is 1.5 times that in blood, but the ratio varied as a function of time after ingestion. The concentration of 8-MOP in serum was compared with that in suction blisters immediately after irradiation was administered to 20 patients with psoriasis and who were receiving PUVA (*10*); the ratio of 8-MOP concentration in serum compared with the fluid from the blisters ranged from 0.06 to 1.54. Thus individual differences in 8-MOP distribution in skin and perhaps other organs may account for some of these variations in 8-MOP plasma profiles.

Of the psoralens that reversibly bind to plasma proteins in vitro (*28, 29*), 8-MOP was 75 to 84% reversibly bound to serum albumin with 2.4 (*28*) and 0.7 (*29*) binding sites/albumin molecule reported. Some workers have acidified all plasma samples to release 8-MOP from plasma proteins before extraction (*4*). However, we and others found almost complete 8-MOP extraction from plasma or serum into the organic phase without performing such a step (*13*). Individual differences in plasma protein binding may affect drug distribution, but they cannot account for differences in plasma profiles.

Low plasma levels may explain a poor therapeutic response in some persons (*14*). Patients with peak levels greater than 65 ng/ml had a history of a good therapeutic response; 1 with a peak 8-MOP level of 29 ng/ml had a moderate therapeutic response, and another with little to no absorption received no benefit from PUVA therapy (*3*). Wagner et al. (*17*) reported low 8-MOP plasma levels and low area-under-the curve values in PUVA-treated patients who presented problems. Their results suggest that the 8-MOP threshold for an erythematous response may approximate its therapeutic threshold. Analyses of 8-MOP plasma profiles may thus be useful in evaluations of patients who respond poorly to PUVA therapy.

We found that the skin phototoxic responses to UV irradiation were maximal 3 hours after ingestion of the drug and significant at 2 hours; however, 8-MOP plasma levels were greatest at 2 hours (*14*). Other investigators (*27*) detected that skin response is greatest at 1 hour, with 8-MOP plasma levels greatest from 1/2 to 1 hour after ingestion of a different formulation of the drug. The time of greatest skin sensitivity probably indicates the time of greatest cutaneous concentration of drug. Equilibrium between 8-MOP concentrations in skin and blood probably requires no more than 1 hour.

No more than one-half of the PUVA-treated patients in 1 of our studies had maximum photosensitivity or peak 8-MOP plasma levels at 2 hours (*14*). Two hours after 8-MOP ingestion, however, is the recommended time for irradiation in the most widely used PUVA protocol (*15*). Variations in 8-MOP absorptive patterns and plasma-to-skin distribution times may account for some of the poor responses to PUVA therapy observed in clinical trials. Peak 8-MOP plasma levels were found at 2 hours in most patients with a history of an adequate therapeutic response in 1 study, but one-half of those presenting problems had deviations in their peak plasma levels (*17*). Of the poor responders to PUVA therapy, 50% had abnormal plasma profiles with later peak levels, i.e., at 5.1 to 8 hours (*11*). Determination of 8-MOP plasma profiles or analyses of MPD profiles may be useful in the evaluation of patients who respond poorly to therapy because of rapid or slow absorption and distribution of adequate plasma concentrations of drug. Improvement in some of the patients who experienced difficulty can be achieved by a shift of the therapeutic UVA irradiation to the peak of the 8-MOP plasma level (*17*).

A general trend in all the time courses is the inverse relationship between 8-MOP plasma levels and MPD of the radiation administered. We observed that the 8-MOP level falls as the MPD rises (*14*). With adjustment for delays in distribution, the converse also appears to be true. Swanbeck et al. (*30*) described a negative linear correlation between the log of the 8-MOP plasma concentration and the MPD in the cumulative data from 5 volunteers. Because of extensive variation in the skin responses of our patient population, we examined each patient individually. We found that the $\log_e$ (MPD) may be inversely proportional to the $\log_e$ (8-MOP). The product of these 2 parameters yields a constant K_t after ingestion and an average value K over all times (*14*): $\log_e \text{MPD} \approx 1/\log_e (\text{8-MOP})_{\text{plasma}}$; constant $K = \log_e \text{MPD} \times \log_e (\text{8-MOP})_{\text{plasma}}$.

The value of K varies from individual to individual and may be influenced by factors affecting absorption, distribution, and metabolism of the drug and by skin type and degree of skin pigmentation. The average value of K is greater for patients of darker skin types: K values averaged 11.9 for skin type III, 9.4 for skin type II, and 8.4 for skin type I. The value of K for each individual might be useful in the prediction of skin response to irradiation given a known plasma concentration of 8-MOP.

REFERENCES

(*1*) Blais J, Dubertret L, Gaboriau F, et al: Fluorescence detection of an 8-methoxypsoralen metabolite in human interstitial fluid. Photochem Photobiol 35:423–426, 1982

(*2*) Steiner I, Prey T, Washuttl J, et al: Serum levels of 8-methoxypsoralen 2 hours after oral administration. Acta Derm Venereol (Stockh) 58:185–186, 1978

(*3*) Herfst MJ, Koot-Gronsveld EA, DeWolff FA: Serum levels of 8-methoxypsoralen in psoriasis patients using a new fluorodensitometric method. Arch Dermatol Res 262:1–6, 1978

(*4*) Chakrabati SG, Gooray DA, Kenney JA: Determination of 8-methoxypsoralen in plasma by scanning fluorometry after thin-layer chromatography. Clin Chem 24: 1155–1157, 1978

(*5*) Gazith J, Schaeffer H: 8-Methoxypsoralen: Its isolation and gas chromatographic determination from aqueous solutions and serum. Biochem Med 18:102–109, 1977

(*6*) Ehrsson H, Eksborg S, Wallin I, et al: Determination of 8-methoxypsoralen in plasma by electron capture gas chromatography. J Chromatogr 140:157–164, 1977

(*7*) Schmid J, Koss FW: Rapid sensitive gas chromatographic analysis of 8-methoxypsoralen in human plasma. J Chromatogr 146:498–502, 1978

(*8*) PUGLISI CV, DESILVA JA, MEYER JC: Determination of 8-methoxypsoralen, a photoactive compound, in blood by high pressure liquid chromatography. Anal Lett 10:39–50, 1977

(*9*) THUNE P, VOLDEN G: Photochemotherapy of psoriasis with relevance to 8-methoxypsoralen plasma level and low intensity irradiation. Acta Derm Venereol (Stockh) 57:351–355, 1977

(*10*) HERFST MJ, EDELBROCK PM, DEWOLFF FA: Determination of 8-methoxypsoralen in suction blister fluid and serum by liquid chromatography. Clin Chem 26:1825–1828, 1980

(*11*) STEVENSON IH, KENICER KJ, JOHNSON BE, et al: Plasma 8-methoxypsoralen concentrations in photochemotherapy of psoriasis. Br J Dermatol 104:47–51, 1981

(*12*) TASKINEN J, VAHVELAINEN N, NORE P: Determination of trioxsalen in human plasma in the picogram range by glass capillary gas chromatography mass spectrometry. Biomed Mass Spectrom 7:556–559, 1980

(*13*) LJUNGGREN B, CARTER DM, ALBERT J, et al: Plasma levels of 8-methoxypsoralen determined by high-pressure liquid chromatography in psoriatic patients ingesting drug from two manufacturers. J Invest Dermatol 74:59–62, 1980

(*14*) GOLDSTEIN DP, CARTER DM, LJUNGGREN B, et al: Minimal phototoxic doses and 8-MOP plasma levels in PUVA patients. J Invest Dermatol 78:429–433, 1982

(*15*) WOLFF K, GSCHNAIT F, HÖNIGSMANN H, et al: Phototesting and dosimetry for photochemotherapy. Br J Dermatol 96:1–10, 1977

(*16*) KLIGMAN AM, GOLDSTEIN FP: Oral dosage in methoxsalen phototoxicity. Arch Dermatol 107:548–550, 1973

(*17*) WAGNER G, HOFMANN C, BUSCH U, et al: 8-MOP plasma levels in PUVA problem cases with psoriasis. Br J Dermatol 101:285–292, 1979

(*18*) GAZITH J, SCHALLA W, SCHAEFER H: 8-Methoxypsoralen: Gas chromatographic determination and serum kinetics. Arch Dermatol Res 263:215–222, 1978

(*19*) EHRSSON H, NILSSON S, EHRNEBO M, et al: Effect of food on kinetics of 8-methoxsalen. Clin Pharmacol Ther 25:167–171, 1979

(*20*) BUSCH U, SCHMID J, KOSS FW, et al: Pharmacokinetics and metabolite pattern of 8-methoxypsoralen in man following oral administration as compared to the pharmacokinetics in rats and dogs. Arch Dermatol Res 262:255–265, 1978

(*21*) WULF HC, HART J: Distribution of tritium labelled 8-methoxypsoralen in the rat, studied by whole body autoradiography. Acta Derm Venereol (Stockh) 59:97–103, 1979

(*22*) PATHAK MA, DALL'ACQUA F, RODIGHIERO G, et al: Metabolism of psoralens. J Invest Dermatol 62:347, 1974

(*23*) PATHAK MA, MANDULA B, NAKAYAMA Y, et al: Cutaneous photosensitization and in vivo metabolism of psoralens. J Invest Dermatol 64:279, 1975

(*24*) STEINER I, PREY T, GSCHNAIT F, et al: Serum level profiles of 8-methoxypsoralen after oral administration. Arch Dermatol Res 259:299–301, 1977

(*25*) STOLK L, KAMMEYER A, CORMANE RH, et al: Serum levels of 8-methoxypsoralen: Difference between two oral methods of administration. Br J Dermatol 103:417–420, 1980

(*26*) KREUTER J, HIGUCHI T: Improved delivery of methoxsalen. J Pharm Sci 68:451–452, 1979

(*27*) THUNE P: Plasma levels of 8-methoxypsoralen and phototoxicity studies during PUVA treatment of psoriasis with meladinin tablets. Acta Derm Venereol (Stockh) 58: 149–151, 1978

(*28*) VERONESE FM, BEVILACQUA R, SCHIAVON O, et al: Drug protein interaction: Plasma protein binding of furocoumarins. Farmaco [Sci] 34:3–12, 1979

(*29*) ARTUC M, STUETTGEN G, SCHALLA W, et al: Reversible binding of 5 and 8-methoxypsoralen to human serum proteins (albumin) and to epidermis in vitro. Br J Dermatol 101:669–677, 1979

(*30*) SWANBECK G, EHRSSON H, EHRNEBO M, et al: Serum concentration and phototoxic effect of methoxsalen in patients with psoriasis. Clin Pharmacol Ther 25:478–480, 1979

DISCUSSION

H. Wulf: In the literature it seems that the concentration in serum is much higher than when measured in plasma, nearly 50% higher if you take all the studies and put them together. Have you any comment on that?

D. M. Carter: In our experience with parallel samples and our assay, we found comparable levels in whole blood, serum, or plasma. It is impossible to compare results from place to place.

A. Kornhauser: Many different commercial sources of psoralens are now available. To verify the purity and authenticity of the psoralen derivative in question, I recommend that each lot received be analyzed by methods in addition to TLC, such as gas chromatography, mass spectrometry, and nuclear magnetic resonance.

Psoralen and Ultraviolet A Effects on Epidermal Ornithine Decarboxylase Induction and DNA Synthesis in the Hairless Mouse[1, 2]

Nicholas J. Lowe, Michael J. Connor, Elizabeth S. Cheong, Patricia Akopiantz, *and* James H. Breeding[3]

ABSTRACT—8-Methoxypsoralen (8-MOP), 3-carbethoxypsoralen (3-CP), and 5-methoxypsoralen (5-MOP), with and without ultraviolet A (UVA), were compared as inducers of epidermal ornithine decarboxylase (ODC) and modulators of epidermal DNA synthesis in vivo in female Skh:hairless-1 albino mice. Both 8- and 5-MOP plus UVA induced epidermal ODC. Peak ODC activity was induced 24 hours after treatment, and ODC activity was still elevated at 48 hours. These same treatments also suppressed epidermal DNA synthesis 4 hours after treatment, as measured by tritiated thymidine incorporation. The psoralen 3-CP, which lacks the DNA cross-linking ability of 8- and 5-MOP and can only form DNA monoadducts, failed to induce epidermal ODC either alone or with UVA and stimulated rather than suppressed incorporation of the tritiated thymidine. — Natl Cancer Inst Monogr 66: 73–76, 1984.

Many treatments used in the therapy of psoriasis have been shown to suppress epidermal DNA synthesis temporarily (*1, 2*). Different forms of phototherapy have been used in the treatment of psoriasis, including coal tars and UVB (*3*) and more recently psoralens and longwave UVA (*4*). These therapies may cause phototoxic skin responses and are possibly carcinogenic to the skin in man (*5*). Both 8- and 5-MOP plus UVA are known cutaneous carcinogens in mice (*6, 7*). Also 8- and 5-MOP are bifunctional furocoumarins and have the ability to form interstrand cross-links in DNA in the presence of UVA (*8*). In a preliminary study (*9*), 3-CP appeared to be effective in a small group of psoriasis patients. Because of the substituent at the 3-position, 3-CP can only form monoadducts with DNA. When combined with UVA, it is noncarcinogenic in mice (*9*).

We have studied the comparative effects of these 3 psoralens with and without UVA on 2 events associated with an abnormal proliferative response: induction of epidermal ODC activity and changes in epidermal DNA synthesis.

The induction of epidermal ODC has been suggested as a biochemical marker for tumor-promoting agents (*10*). Carcinogenic wavelengths of UVB and UVC can also induce epidermal ODC (*11, 12*). Suppression of epidermal DNA synthesis, measured by [^{3}H]dThd incorporation into the epidermis of hairless mice (*1, 2*), is an assay possibly predictive of antipsoriatic effects and detects agents that induce or inhibit epidermal DNA synthesis.

In this paper, we compare the effects of topical and oral 8-MOP, 5-MOP, and 3-CP with and without UVA on epidermal ODC activity and DNA synthesis.

MATERIALS AND METHODS

Mice.—All experiments were performed on female Skh: hairless-1 (hairless albino) mice supplied by Temple University (Philadelphia, Pa.) and used 2 weeks after arrival. Mice were 10–12 weeks old at the time, weighed 25–30 g, were housed prior to use in a windowless room, and fed Purina Laboratory mouse chow and water ad libitum.

Administration of psoralens.—Doses of 0–25 mg psoralens/kg, dissolved by slight warming and extensive vortical mixing, were administered orally as a solution in corn oil. The mice were irradiated with UVA 2 hours later.

For topical administration, psoralens were dissolved in acetone. The solutions were applied to the dorsal skin of the mice with a variable pipette with a disposable tip; volumes of 200 μl could be applied quite readily in this manner. The mice were irradiated 1 hour after topical applications.

Irradiation of mice.—Mice, housed in retaining cages, were irradiated with UVA from a bank of FT40T12/PUVA lamps mounted 15 cm above the mouse dorsal surface. The radiation was continually monitored by an IL700 radiometer and an IL720 photodosimeter with an appropriate precalibrated sensor. A typical irradiation with 5 J/cm^2 took approximately 45 minutes to deliver.

Evaluation of phototoxic skin erythema and edema.—We noted visible skin erythema and estimated edema by measuring the double skin-fold thickness of the dorsal skin of the mice using a ratchet micrometer at 24 and 48 hours following UVA irradiation.

Preparation of mouse epidermis for ornithine decarboxylase assay.—Mice were killed by cervical dislocation and the back skin was removed. The epidermis was separated from the dermis by a brief heat treatment in a water bath at 55° C for 30 seconds and cooled in an ice-water bath. The skin was placed flat on a glass plate and, by scraping with a scalpel blade, we separated the epidermis.

ABBREVIATIONS: UVA = UV radiation at 320–400 nm; UVB = UV radiation at 290–320 nm; UVC = UV radiation at 250–290 nm; 8-MOP = 8-methoxypsoralen; 5-MOP = 5-methoxypsoralen; 3-CP = 3-carbethoxypsoralen; ODC = ornithine decarboxylase; dThd = thymidine; PUVA = psoralen plus UVA; J = joule(s).

[1] Presented at the Conference on Photobiologic, Toxicologic, and Pharmacologic Aspects of Psoralens held in Research Triangle Park, North Carolina, March 1–3, 1982.

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Assay of ornithine decarboxylase.—Epidermal scrapings were homogenized in 1 ml ice-cold buffer, and ODC activity was determined by measurement of the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine as described in (*11, 12*).

All the assays were performed in sealed Pyrex tubes, incubated at 37° C for 1 hour. We collected $^{14}CO_2$ directly into a scintillation vial containing NCS and fitted to each tube by a side arm and a rubber stopper. Injection of 0.5 ml 2-*M* citric acid through a sleeve stopper in the mouth of the tube terminated the reactions. Results were expressed as nanomoles CO_2 released per hour per milligram protein.

Measurement of epidermal DNA synthesis by [^{3}H]thymidine incorporation.—This assay, which measures epidermal fixation of [^{3}H]dThd per unit area of epidermis, is a modification of the method of Otani et al. (*13*). Mice were given ip injections of 250 μCi [^{3}H]dThd 1 hour before being killed by cervical dislocation. The dorsal skin was removed and flattened on a glass plate, and the epidermis was scored with a 17-mm punch. The whole skin was immersed in water at 55–60° C for 30 seconds, cooled in ice water, and then the epidermis was scraped from within the areas marked out by the punch. The epidermal disk scrapes were placed in embedding bags and processed in an Autotechnicon automatic tissue processor for removal of the unincorporated [^{3}H]dThd. Residual radioactivity in the disks was determined in a scintillation counter. Results were expressed as counts per minute per disk and related to the percentage incorporation of [^{3}H]dThd into the epidermis of untreated or placebo-treated controls.

RESULTS

Epidermal Ornithine Decarboxylase Induction by Topical Psoralens Plus Irradiation

The results of the effects of 200 μl of 1% each of 8- and 5-MOP or 2% 3-CP applied 1 hour before 5 J UVA/cm^2 are given in figure 1. The mice were killed 0, 4, 24, and 48 hours after irradiation. Significant ODC activity was induced at 24 hours, remaining elevated at 48 hours by 8- or 5-MOP plus UVA. No comparable increase in ODC activity was noted in the 3-CP- and UVA-treated mice at any time.

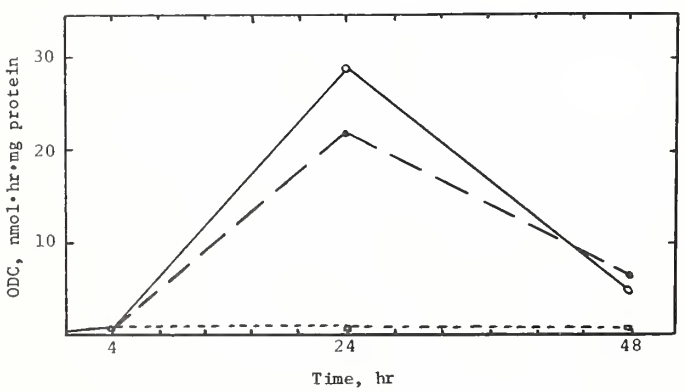

FIGURE 1.—Time course of epidermal ODC induction by topical psoralens plus UVA. Mice were treated with 200 μl 1% 8-MOP plus 5 J UVA/cm^2 (—○—○—), 200 μl 1% 5-MOP plus 5 J UVA/cm^2 (—●—●—), or 200 μl 2% 3-CP plus 5 J UVA/cm^2 (---□---□---). Five mice/group were killed at the stated times, and the epidermal ODC activity was measured. Control groups (each psoralen alone and UVA alone) gave values below those for the 3-CP plus UVA-treated mice.

TABLE 1.—*Induction of epidermal ODC after oral psoralen and UVA*[a]

Treatment	Epidermal ODC activity, nmol·hr^{-1}·mg^{-1} protein
UVA only	0.06±0.02
8-MOP + UVA	1.40±0.20
3-CP + UVA	0.03±0.01

[a] Five mice/group were dosed with 25 mg oral psoralen/kg body weight and irradiated 2 hr later with 5 J UVA/cm^2. They were killed 24 hr later.

Effects of Oral Psoralen and Irradiation on Epidermal Induction

Epidermal ODC activity was detected 24 hours after 8-MOP plus UVA treatment but not after 3-CP plus irradiation (table 1).

Effects of Psoralen Plus Irradiation on Epidermal DNA Synthesis

Two amounts of UVA irradiation (1 and 5 J/cm^2) were given 1 hour after the topical psoralen. Treatment with 8- and 5-MOP plus UVA reduced epidermal DNA synthesis at 4 hours. No inhibitory effects were shown by 3-CP plus UVA treatment on epidermal DNA synthesis at this time; on the contrary, incorporation of [^{3}H]dThd was increased (fig. 2; table 2). Epidermal DNA synthesis was also suppressed at 4 hours after oral dosing with 25 mg 8-MOP/kg plus UVA.

Erythema and Edema Induction by Psoralen Plus Irradiation

Topical 8-MOP and 5-MOP plus UVA produced visual erythema and a marked increase in the dorsal skin-fold thickness, but 3-CP plus UVA showed no effect (table 3).

TABLE 2.—*Effect of topical psoralen plus UVA on [^{3}H]dThd incorporation into epidermal disks*

Treatment		Mean cpm/disk ± SD	P[b]
Psoralen[a]	UVA, J/cm^2		
Vehicle	5	1,439±842	—
8-MOP	1	191± 45	<0.01
8-MOP	5	154±180	<0.025
3-CP	1	2,381±489	>0.05
3-CP	5	1,590±527	>0.05
Vehicle	5	1,626±219	—
5-MOP	1	85± 22	<0.0005
5-MOP	5	28± 8	<0.0005

[a] Psoralen dose = 50 μl of 1% psoralen in acetone 1 hr before UVA; mice were killed 4 hr after UVA.

[b] P values for differences between psoralen plus UVA groups vs. vehicle plus UVA groups were obtained with the t test.

TABLE 3.—*Erythema and edema induced by topical psoralen and UVA*

Treatment[a]	Erythema	Dorsal skin-fold thickness, mm	Erythema	Dorsal skin-fold thickness, mm
	24 hr		48 hr	
8-MOP + UVA	+	1.32±0.43	+	1.36±0.04
5-MOP + UVA	+	1.68±0.31	+	1.15±0.07
3-CP + UVA	0	0.84±0.08	0	0.78±0.09
UVA	0	0.75±0.08	0	0.82±0.07

[a]We applied 200 μl of 1% 8- or 5-MOP or 2% 3-CP topically 1 hr before 5 J of UVA/cm^2.

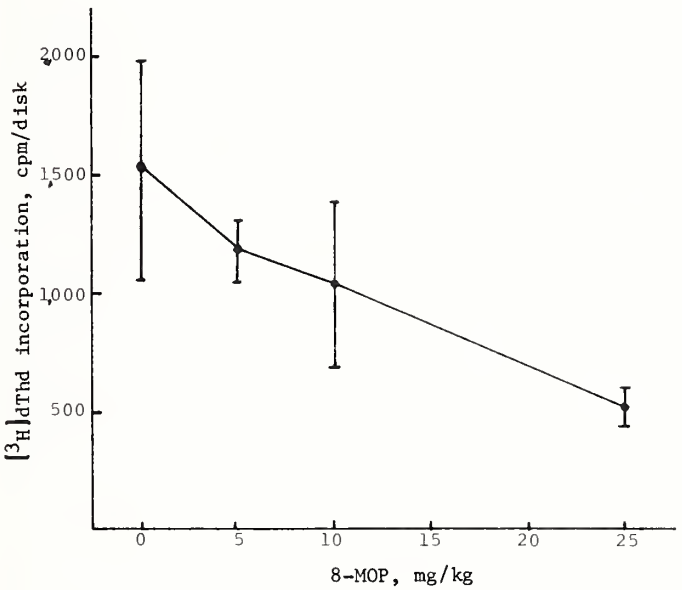

FIGURE 2.—Effect of 8-MOP dose on the inhibition of [^{3}H]dThd incorporation into epidermal disks by oral 8-MOP plus UVA. Mice were fed 8-MOP in corn oil by gavage 1 hr before 5 J UVA/cm^2 and killed 4 hr later. *Each point* represents the mean ± SD cpm/disk obtained from 5 mice. cpm = counts per minute.

DISCUSSION

The 3 psoralens plus UVA have different abilities to influence epidermal ODC and DNA synthesis. The ODC, which forms putrescine by the decarboxylation of ornithine, catalyzes the first and probably the rate-limiting step in the biosynthesis of the polyamines spermidine and spermine (*14*). The activity of ODC and the levels of the polyamines are elevated in various hyperproliferative systems (*15*). Application of the tumor-promoting agent 12-*O*-tetradecanoylphorbol-13-acetate to mouse skin leads to a great increase in epidermal ODC activity, and this induction has been suggested to be essential for skin tumor promotion (*10*).

Those psoralens (8- and 5-MOP) known to be carcinogenic to mouse skin (*6, 7*) are capable of marked induction of epidermal ODC. However, 3-CP apparently is noncarcinogenic (*9*) in mouse skin and failed to induce epidermal ODC.

We (*1, 2*) have shown that measurement of suppression of epidermal DNA synthesis in the hairless mouse is a useful predictive screen for antipsoriatic drugs and chemicals. Both 8- and 5-MOP plus UVA show significant antipsoriatic effects in controlled clinical studies (*4, 16*). It remains to be seen if 3-CP plus UVA is as effective an antipsoriatic therapeutic agent as are 8- or 5-MOP plus UVA in carefully controlled comparison studies of patients with psoriasis. The results presented here suggest that 3-CP would not be as efficacious as 8- or 5-MOP in PUVA therapy.

The mechanisms of PUVA carcinogenesis and its antipsoriatic effects are unknown but may involve several cellular processes. Both 8- and 5-MOP are photoactivated by UVA and can act as bifunctional agents capable of cross-linking adjacent strands of DNA (*8*). Also photoactivated by UVA but because the 3-4 reaction site is blocked, 3-CP can only form monoadducts with DNA (*9*), and these adducts may be more easily repaired than the cross-links induced by the bifunctional agents (*9*). This may explain the increased incorporation of [^{3}H]dThd in mouse epidermis observed after 3-CP plus UVA treatment, attributable to DNA repair mechanisms, rather than the pronounced suppression of [^{3}H]dThd incorporation due to inhibition of DNA synthesis found after 8- or 5-MOP plus UVA treatment.

In summary, these 2 phototoxic psoralens plus UVA were active inducers of epidermal ODC and suppressed epidermal DNA synthesis. The nonphototoxic 3-CP had no effect.

REFERENCES

(*1*) LOWE NJ, STOUGHTON RB, MCCULLOUGH JL, et al: Topical drug effects on normal and proliferating epidermal cell models. Comparison with responses in psoriatics. Arch Dermatol 117:394–398, 1981

(*2*) LOWE NJ, BREEDING J: Anthralin. Different concentration effects on epidermal cell DNA synthesis rates in mice and clinical responses in human psoriasis. Arch Dermatol 117:698–700, 1981

(*3*) GOECKERMAN WH: Treatment of psoriasis. Northwest Med 24:229–231, 1925

(*4*) PARRISH JA, FITZPATRICK TB, TANENBAUM L: Photochemotherapy of psoriasis with oral methoxsalen and long wave ultraviolet light. N Engl J Med 291:1207–1211, 1974

(*5*) STERN RS, THIBODEAU LA, KLEINERMAN RA, et al: Risk of cutaneous carcinoma in patients with oral methoxsalen photochemotherapy for psoriasis. N Engl J Med 300: 809–813, 1979

(6) SONG P-S, TAPLEY KJ: Photochemotherapy and photobiology of psoralens. Photochem Photobiol 29:1177–1197, 1979

(7) ZAJDELA F, BISAGNI E: 5-Methoxypsoralen, the melanogenic additive in suntan preparations, is tumorigenic in mice exposed to 365 nm UV radiation. Carcinogenesis 2:121–127, 1981

(8) MUSAJO L, RODIGHIERO G, CAPORALE G, et al: Photoreactions between skin-photosensitizing furocoumarins and nucleic acids. *In* Sunlight and Man: Normal and Abnormal Photobiologic Responses (Fitzpatrick TB, Pathak MA, Harber LC, et al, eds). Tokyo: Univ Tokyo Press, 1974, pp 369–387

(9) DUBERTRET L, AVERBECK D, ZAJDELA F, et al: Photochemotherapy of psoriasis using 3-carbethoxypsoralen, a noncarcinogenic compound in mice. Br J Dermatol 101:379–389, 1978

(10) O'BRIEN TG: The induction of ornithine decarboxylase as an early, possibly obligatory event in mouse skin carcinogenesis. Cancer Res 36:2644–2653, 1976

(11) LOWE NJ, VERMA AK, BOUTWELL RK: Ultraviolet light induces epidermal ornithine decarboxylase. J Invest Dermatol 71:417–418, 1978

(12) LOWE NJ: Ultraviolet light and epidermal polyamines. J Invest Dermatol 77:147–153, 1981

(13) OTANI AS, GANGE RW, WALTER JW: Epidermal DNA synthesis, a new disk technique for evaluating incorporation of tritiated thymidine. J Invest Dermatol 75:375–378, 1980

(14) JANNE J, POSO H, RAINE A: Polyamines in rapid growth and cancer. Biochim Biophys Acta 473:241–293, 1978

(15) TABOR CW, TABOR H: 1,4-Diaminobutane (putrescine), spermidine and spermine. Annu Rev Biochem 45:285–306, 1976

(16) HÖNIGSMANN H, JASCHKE E, GSCHNAIT F, et al: 5-Methoxypsoralen in photochemotherapy of psoriasis. Br J Dermatol 101:369–378, 1979

DISCUSSION

M. Pathak: Certainly, the problem in some of these biologic experiments is the phototoxicity of these compounds. The eye is one way to detect erythema reaction, but it is most unpredictable. Dr. Lowe provides an additional measure of psoralen toxicity in the skin with ODC and DNA synthesis levels.

Dr. Lowe, in your protocol, you went up to 1% psoralen levels, but did you go lower than that because you do not need that high a concentration for phototoxicity in animal skin?

N. J. Lowe: We looked at lower concentrations, and certainly you can show significant ODC induction with 0.1% 8- and 5-MOP but not with 2% 3-CP concentrations. The reason we selected the 1% concentration of the latter psoralen is that that concentration was apparently effective clinically in psoriasis.

Psoralen Pharmacology: Studies on Metabolism and Enzyme Induction[1]

David R. Bickers[2] *and* Madhu A. Pathak[3]

ABSTRACT—Psoralens, tricyclic furocoumarins with potent photosensitizing properties in the skin, are now widely used in the treatment of several dermatologic diseases. In this study, the metabolism of 4,5′,8-trimethylpsoralen (TMP) and 8-methoxypsoralen (8-MOP) was studied in mouse liver. Orally administered TMP is transformed into several metabolites, the major one of which is 4,8-dimethyl-5′-carboxypsoralen (DMCP) in both humans and mice. Orally administered 8-MOP is metabolized into at least 5 fluorescent moieties, including 8-hydroxypsoralen, the 4′5′-dihydro-diol of 8-MOP, and furocoumaric acid. The effects of 3 psoralens, 8-MOP, TMP, and isopsoralen (angelicin) on hepatic microsomal drug-metabolizing enzymes and cytochrome P-450 were assessed in mice and rats. Administered orally to CD-1 mice daily for 6 days, 8-MOP caused twofold to threefold increases in hepatic aryl hydrocarbon hydroxylase (AHH), ethylmorphine *N*-demethylase, and cytochrome P-450. The absorbance maximum of the induced cytochrome was at 450 nm. Aniline hydroxylase activity was unchanged. Chronic administration of 8-MOP to Skh:hairless-1 mice caused significant enhancement of hepatic ethylmorphine *N*-demethylase and cytochrome P-450 but had no effect on AHH, whereas chronically administered TMP had no significant effect on any of these parameters. Isopsoralen and TMP administered orally to CD-1 mice daily for 6 days had no effect on any of these liver enzymes or on hepatic P-450, but 8-MOP administered daily for 6 days to Sprague-Dawley rats caused a greater than fourfold enhancement of AHH and greater than twofold enhancement of ethylmorphine *N*-demethylase and cytochrome P-450. These studies indicate that photosensitizing psoralens are substrates for and can induce the activity of the hepatic mixed function oxidase system. — Natl Cancer Inst Monogr 66: 77–84, 1984.

Psoralens are furocoumarin compounds present in numerous plants found throughout the world (*1*). They are also widely used in the treatment of a number of dermatologic disorders, including vitiligo, psoriasis, and mycosis fungoides, because of their photosensitizing properties (*2–4*). Psoralens are capable of absorbing UV radiation and the photoexcited molecule is capable of forming C_4-cycloaddition products with pyrimidine bases of DNA (*5*). Both fluorescent and nonfluorescent monoadducts and interstrand cross-links are formed, and these photoaddition products are thought to result in the inhibition of DNA replication (*6*). Furthermore, the combination of psoralens and UV radiation results in the enhancement of melanogenesis. These properties have led to the general use of topically applied or systemically administered psoralens followed by exposure of the skin to UVA, i.e., PUVA therapy, in treating several disorders of the skin (*7*).

Despite their increasing use in practice by dermatologists and oncologists, relatively little is known about the pharmacology of these drugs. Although gastrointestinal absorption following oral administration is generally complete within 1–3 hours, absorption is more prolonged and incomplete in some individuals (*8*). Circulating plasma levels of the psoralen range from 25 to 275 ng/ml plasma.

Despite the availability of existing knowledge regarding the absorption and distribution of the psoralens, much less information exists concerning their metabolism and effects on the hepatic microsomal cytochrome P-450-dependent monooxygenase that is responsible for the metabolism of most xenobiotics (*9*).

Previous studies have shown that TMP is metabolized to DMCP in humans and mice (*10*). Our studies were designed to assess the metabolism of TMP and 8-MOP and to determine the effects of these compounds as well as isopsoralen on the hepatic mixed function oxidase. Our data indicate that TMP and 8-MOP are metabolized to a number of derivatives and are inducers of drug-metabolizing enzymes in mammalian liver.

MATERIALS AND METHODS

Materials.—The TMP, received in tritiated form from New England Nuclear Corporation (Boston, Mass.), was purified as described in (*11*); it had a specific activity of 40 μCi/μm and was used routinely at about 0.3 μCi/μm. Tritiated DMCP was obtained from the urine of CD-1 mice receiving [^{3}H]TMP as described in (*10*). Nonradioactive TMP and 8-MOP were gifts from Elder Pharmaceuticals (Bryan, Ohio) and were used after repeated crystallization from ethanol. Ether extractions were performed with anhydrous ether from the Mallinckrodt Chemical Company (St. Louis, Mo.). The NADPH for the tissue preparations and BP and MCA were obtained from Sigma Chemical Company (St. Louis, Mo.). Aroclor 1254 was a gift from the Monsanto Corporation (St. Louis, Mo.).

Tissue preparations for metabolic studies.—Male CD-1 from the Charles River Breeding Laboratories, weighing 25–30 g, were killed by cervical dislocation. The liver or

Abbreviations: PUVA = psoralen plus UV radiation at 320–400 nm; TMP = 4,5′8-trimethylpsoralen; DMCP = 4,8-dimethyl, 5′-carboxypsoralen; 8-MOP = 8-methoxypsoralen; BP = benzo[*a*]pyrene; 3-MCA = 3-methylcholanthrene; TLC = thin-layer chromatography; R_F = retardation factor; AHH = aryl hydrocarbon hydroxylase; 3-OHBP = 3-hydroxybenzo[*a*]pyrene.

[1] Presented at the Conference on Photobiologic, Toxicologic, and Pharmacologic Aspects of Psoralens held in Research Triangle Park, North Carolina, March 1–3, 1982.

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kidney was removed, weighed, and placed in a glass Teflon Potter-Elvehjem homogenizer with 3 vol of either Tris–sucrose buffer (0.25 *M* sucrose and 0.05 *M* Tris-chloride, pH 7.4) or phosphate–sucrose buffer (0.25 *M* sucrose, 0.01 *M* sodium phosphate, pH 7.4, 0.001 *M* EDTA, and 0.05 *M* nicotinamide).

Guinea pig epidermal homogenates were prepared according to a procedure described by Pathak and Krämer (*11*). Backs of female guinea pigs weighing 400–600 g were epilated 24–48 hours before they were killed with ether anesthesia. The epidermis was scraped from the stretched skin and homogenized with 10 vol of sucrose–phosphate buffer. To prepare microsomes, we centrifuged homogenates at 10,000×*g* for 20 minutes; the resulting supernatant fraction was spun at 105,000×*g* for 60 minutes. Microsomes were resuspended in the homogenizing buffer. Protein was determined by the procedure of Lowry et al. (*12*) after precipitation by 5% trichloroacetic acid.

Reaction of tissue preparations with psoralens.—In 2.5 ml, the reaction mixture contained 50 m*M* sodium phosphate, pH 7.4; 3 m*M* glucose 6-phosphate; 4 m*M* magnesium chloride; 0.16 m*M* NADP; 0.06 m*M* NAD; 0.4 m*M* EDTA; 12 m*M* nicotinamide; 0.3 U glucose-6-phosphate dehydrogenase; 350–450 nmol TMP or 8-MOP. The reaction took place at 37° C for 30 minutes and was vigorously shaken in 20-ml glass scintillation counting vials after 5-minute preincubation in the absence of tissue. The reaction was terminated by the addition of 10 ml anhydrous diethyl ether. In control reactions, we added the psoralen or tissue after the ether. After vigorous shaking by hand, the ether layer was removed and saved. Then 0.3 ml of 1 *N* HCl was added to the aqueous layer to give a pH of about 2, and the extraction was repeated with 10 ml of additional ether. The ether layers were combined, evaporated to dryness, and the residues dissolved in a small volume (0.2 ml) of ethanol for TLC and absorption spectroscopy.

The transformation in vitro of [^{3}H]TMP was quantitated by the chromatograms obtained. We measured the transformation of nonradioactive 8-MOP or TMP by eluting the chromatograms with ethanol and obtaining absorption spectra. At 250 nm and a 1-cm light path, 10 μg TMP/ml has an optical density of 1.4 or $E^1_{250} = 3.20 \times 10^4$.

Thin-layer chromatography.—Four solvent systems with the following composition by volume were used for TLC: solvent A: chloroform:ethylacetate:acetic acid, 6:3:1; solvent B: chloroform:ethylacetate:acetic acid, 5:4:1; solvent C: toluene:acetic acid, 8:2; solvent D: H_2O only. Solvents A, B, and C were used with Bakerflex silica gel IB or IB_2 plates with or without fluorescent indicator; solvent D was used on Bakerflex cellulose plates. The silica gel and cellulose plates were obtained from J. T. Baker Chemicals (Phillipsburg, N.J.). Compounds were detected by their characteristic fluorescence, color, and R_F values when viewed in the dark with longwave (320–400 nm) or shortwave (254 nm) UV-emitting lamps.

Preparation of TMP metabolites.—Metabolic products of TMP were designated M_1, M_2, and M_3 and were prepared from standard TMP incubations with mouse liver homogenate and TLC IB_2 plates for chromatography in solvent A. Fluorescent substances M_1 and M_2 and R_F values of 0.12 and 0.35, respectively, were eluted with ethanol with corresponding bands from control TLC plates. After ethanol evaporation, sometimes, M_1 and M_2 were purified by ether extraction from acidified aqueous solution.

Radioactivity determination.—Tritium was counted in a Model 3300 Tri-Carb liquid scintillation instrument from Packard Instrument Company (Downers Grove, Ill.) with the use of 10 ml Aquasol (New England Nuclear Corp.). Chromatograms were cut into thin strips which were placed in counting vials with 1 ml ethanol. Aquasol was added after shaking the vials for 15 minutes. Counting efficiency was about 35%; all data are corrected for background.

Spectroscopy.—The mass spectra were taken on an AEI-9 double focusing mass spectrometer with an electron impact source at 70 electron volts. The source temperature was the minimum required for adequate ion intensity. The UV absorption of ethanolic solutions of the metabolites was recorded between 220 and 400 nm on a Spectronic 505 recording spectrophotometer from Bausch & Lomb, Inc. (Rochester, N.Y.). The activation and emission spectra of ethanolic solutions of the metabolites were obtained on a Model J-48201 spectrophotophosphorimeter for Aminco-Keirs (Silver Spring, Md.).

Treatment of animals for studies of enzyme induction.—Male CD-1, weighing 25–30 g, were treated daily for 6 days by oral administration of 8-MOP, TMP, or isopsoralen at a dose of 0.8 $mg \cdot kg^{-1} \cdot day^{-1}$. Because all these drugs are highly insoluble in water (50 mg/liter), they were suspended in 10% gum acacia (USP) and administered by stomach tube. Controls received vehicle alone. In other experiments, the effects of orally administered 8-MOP were compared with those of typical inducers of drug metabolism such as phenobarbital (75 $mg \cdot kg^{-1} \cdot day^{-1}$ for 3 days in saline) and 3-MCA (40 $mg \cdot kg^{-1} \cdot day^{-1}$ for 3 days in corn oil) in the CD-1 mice. The effects of chronically administered 8-MOP (1.2 $mg \cdot kg^{-1} \cdot day^{-1}$) 5 days/week for 6 months were also evaluated with Skh:hairless-1 mice obtained from Dr. G. Mann, Genetics Division, Skin and Cancer Hospital, Temple University (Philadelphia, Pa.)

We also assessed the effects of 8-MOP in male Sprague-Dawley rats from Holtzman Rat Farm (Madison, Wis.) by suspending it in corn oil by sonication and administering it orally at a dose of 0.8 $mg \cdot kg^{-1} \cdot day^{-1}$ for 6 days. On day 7, the animals were killed. In other studies, the effects of topical application of 8-MOP and 3-MCA on cutaneous drug metabolism were compared in rat skin. The nuchal region of the animals was shaved with clippers and treated with topically applied 8-MOP (50 mg/kg in acetone) or 3-MCA (50 mg/kg) once daily for 3 days; the animals were killed on day 4.

Twenty-four hours after the last treatment the animals were killed by decapitation, and washed hepatic microsomes were prepared as described (*13*). Whole skin homogenates were prepared by techniques routinely used in these laboratories (*14*).

Enzyme induction studies (enzyme assays).—Cytochrome P-450 concentrations in hepatic microsomes were analyzed from the dithionite plus carbon monoxide minus dithionite difference spectrum (*15*) with use of an Aminco Chance DW-2a spectrophotometer from the American Instrument Company (Silver Spring, Md.). Base-line cor-

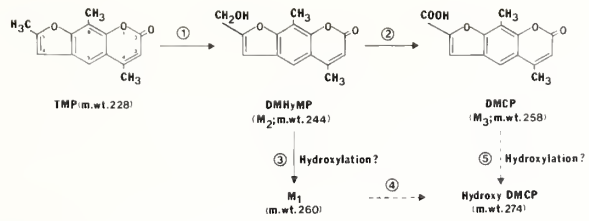

FIGURE 1.—Metabolism of TMP following oral administration of 1.6 mg/kg to humans and 2 mg/mouse. m. wt. = molecular weight; DMHyMP = dimethylhydroxymethylpsoralen. Figure is reproduced with permission of the publisher (*22*).

rection was obtained with a Midan microprocessor (also from Aminco). We used the molar extinction coefficient of 91,000 for the absorbance change between 450 and 490 nm to determine the cytochrome P-450 concentrations and a method described by Alvares and Mannering (*16*) for ethylmorphine *N*-demethylase activity. The formaldehyde formed was estimated colorimetrically (*17*). Aniline hydroxylase activity was assayed by measurement of the formation of *p*-aminophenol by the phenol indophenol method of Imai et al. (*18*) and that of AHH by a modification of the method of Nebert and Gelboin (*19*) as described in (*13*). The quantitation of phenolic BP metabolites was based on comparison of fluorescence to a standard of 3-OHBP.

Spectral binding studies.—Microsomal suspensions containing microsomes equivalent to 100 mg of liver wet weight/ml of 0.1 *M* dipotassium phosphate:potassium biphosphate buffer, pH 7.4, were used. Spectral changes caused by the addition of 8-MOP to liver microsomes were done according to Schenkman et al. (*20*).

RESULTS

Metabolism of 4,5′,8-Trimethylpsoralen and 8-Methoxypsoralen

The metabolic pathway of TMP, shown in figure 1, indicates that biotransformation in the mouse occurs in the liver and appears to involve demethylation at the 5′-position as well as hydroxylation and glucuronate formation. The metabolism of 8-MOP involves formation of at least 5 derivatives as shown in table 1.

With TMP, we succeeded in isolating and characterizing at least 2 major metabolites, 1 of which has been shown to occur in the urine of humans and mice (*21*). This metabolite has a molecular weight of 258.0 and is known as DMCP; it has been synthesized, and its physical, chemical, and photobiologic properties have been examined (*22*). Both the synthetically derived DMCP and the urinary metabolite are identical in every respect (*22*). This major metabolite was observed to be nonphotosensitizing when applied to the skin of human volunteers and depilated albino guinea pigs. The ability of DMCP to photoconjugate with DNA and to form interstrand cross-links was also examined, and the metabolite was poorly conjugated with DNA and a weak cross-linking agent.

Our findings concerning the metabolism of 8-MOP are shown in figure 2. Seven fluorescent metabolites can be identified in the urine of Skh:hairless-1 mice that received oral 8-MOP. The metabolites that have been fairly well characterized are: 1) 8-hydroxypsoralen (A), 2) 4′,5′-dihydrodiol-8-methoxypsoralen (C), 3) open-chain furocoumaric acid in which the lactone ring has been hydrolyzed (D), and 4) unmetabolized 8-MOP (E), which is a minor fraction (5%) of the excreted moieties. Thus hydroxylation, glucuronide formation, epoxidation, and hydrolysis leading to the opening of the lactone ring seem to be the major reaction pathways for biotransformation of 8-MOP.

Comparative Effects of Psoralens on Hepatic Drug Metabolism and Cytochrome P-450 in Mice

Oral administration of isopsoralen (angelicin) and 8-MOP caused significant increases in hepatic microsomal protein, whereas TMP had no such effect (table 2). Also, 8-MOP caused a threefold increase in AHH activity, a twofold increase in ethylmorphine *N*-demethylase activity, and a 50% increase in cytochrome P-450. The absorbance maximum of the 8-MOP-induced hepatic microsomal heme protein was at 450 nm (fig. 3). In contrast, isopsoralen and TMP evoked no statistically significant increases in any of these latter measurements.

Comparative Effects of 8-Methoxypsoralen With Other Typical Inducers of Hepatic Drug-metabolizing Enzymes

Inducers of cytochrome P-450 and hepatic drug-metabolizing enzymes can be divided into at least two broad categories. One group, of which phenobarbital is a typical example, enhances the biotransformation of a large number of substrates and increases a microsomal heme protein with an absorbance maximum at 450 nm. The second group, of which 3-MCA is a typical example, enhances the metabolism of a much smaller number of substrates and induces a microsomal heme protein known as P-448 or P_1-450 that is spectrally and catalytically distinct from P-450 (*23*). We compared the effect of enzyme induction of 8-MOP with that of phenobarbital and 3-MCA in an effort to identify the category of the inducer to which it belongs.

As shown in table 1, both 8-MOP and phenobarbital caused significant increases in hepatic microsomal protein content and in ethylmorphine *N*-demethylase activity, whereas 3-MCA had no such effect. Phenobarbital and 3-MCA significantly enhanced aniline hydroxylase activity, but 8-MOP did not. The 3 compounds significantly increased hepatic microsomal AHH activity. These data

TABLE 1.—*Urinary metabolites isolated from mice receiving oral 8-MOP*[a]

Metabolite	Fluorescence[b]	R_F	Fluorescence[c]
M-1	Light green	0.181	Bluish white
M-2	Bright yellow	0.413	Bright yellow
M-3	Green	0.494	Blue green
M-4	Light yellow	0.05	Light green
M-5	Light green	0.569	Light yellow
(unmetabolized 8-MOP			

[a] Each mouse received 2 mg.
[b] A 254-nm lamp was used.
[c] Wood's light (longwave radiation) was used.

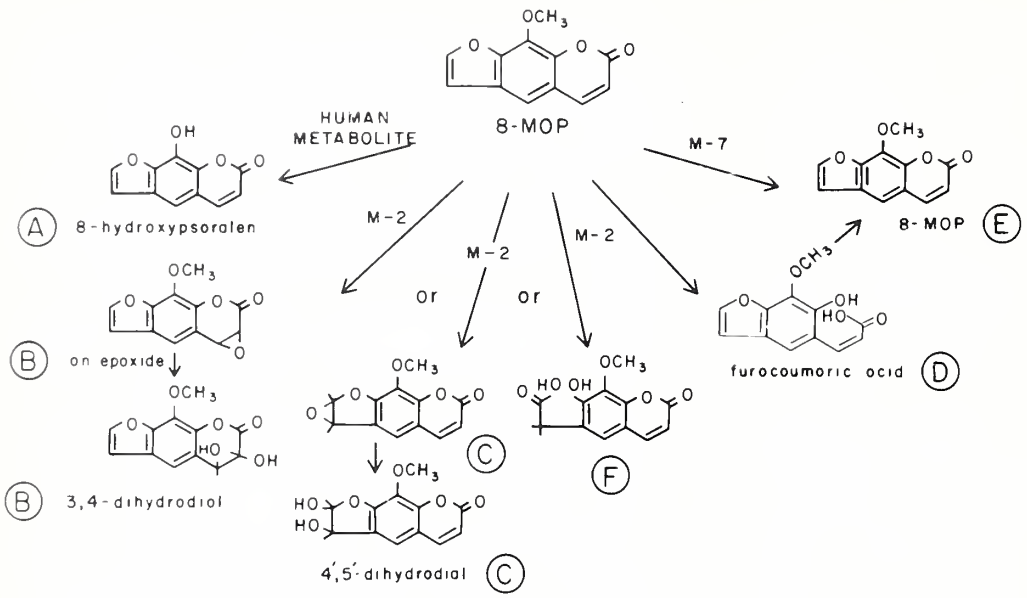

FIGURE 2.—Metabolism of 8-MOP in mice following oral administration of 2 mg. Figure is reproduced with permission of the publisher of *J Invest Dermatol* 79: 201–205, 1982.

indicate that 8-MOP is a phenobarbital type of P-450-inducer in CD-1 mouse liver. In further studies, the spectral characteristics of the cytochrome P-450 induced by 8-MOP were compared with those evoked by phenobarbital and 3-MCA. The carbon monoxide difference spectra of liver microsomes from control mice and from mice treated with 8-MOP, phenobarbital, and 3-MCA are shown in figure 3. As would be expected, liver microsomes from controls and phenobarbital-treated animals showed an absorption maximum at 450 nm, whereas microsomes from 3-MCA-treated animals showed an absorption maximum at 448 nm. Liver microsomes isolated from 8-MOP-treated mice demonstrated a peak absorbance at 450 nm. These data indicate that 8-MOP is a phenobarbital type of heme protein inducer, although its potency as such appears to be considerably less than that of the barbiturates.

Effects of Chronic Administration of 8-Methoxypsoralen and Trimethylpsoralen on Hepatic Drug Metabolism in Hairless Mice

Oral administration of 8-MOP for 6 months evoked a significant increase in ethylmorphine *N*-demethylase activity and in cytochrome P-450 levels but had no effect on AHH activity (table 3). Chronically administered TMP caused no detectable changes in any of these measurements.

Effects of 8-Methoxypsoralen on Hepatic Drug Metabolism in Sprague-Dawley Rats

We conducted these studies with young adult male Sprague-Dawley rats to characterize further the enzyme induction effects of 8-MOP in another widely investigated animal species. Although 8-MOP caused significant increases in liver microsomal AHH and ethylmorphine *N*-demethylase activities and in cytochrome P-450 levels, it had no effect on microsomal protein content or on aniline hydroxylase activity (table 4). Carbon monoxide difference spectra of hepatic microsomes prepared from these animals also confirmed that 8-MOP induced a heme protein with an absorbance maximum at 450 nm. In addition, the ethyl isocyanide difference spectra of microsomes from control and 8-MOP-treated rats showed 455:340 peak ratios of 0.64 and 0.66, respectively (table 4). These ratios are also consistent with a phenobarbital type of induction response.

TABLE 2.—*Comparative effects of orally administered psoralens to CD-1 mice on hepatic cytochrome P-450 levels and drug-metabolizing enzyme activities*[a]

Treatment	Microsomal protein	AHH, pmol 3-OHBP·min^{-1}·mg^{-1} protein	Aniline hydroxylase, nmol peroxidase-antiperoxidase·min^{-1}·mg^{-1} protein	Ethylmorphine *N*-demethylase, nmol formaldehyde·min^{-1} ·mg^{-1} protein	Cytochrome P-450, nmol/mg protein
Control	15.8±0.2	122±7	1.83±0.03	8.43±1.00	1.10±0.04
Isopsoralen	19.6±0.4	117±10	1.68±0.09	7.03±1.33	1.04±0.05
TMP	16.0±0.2	150±10	1.83±0.08	7.13±0.04	1.16±0.02
8-MOP	18.0±0.5[b]	345±4[b]	2.03±0.15	16.60±1.17[b]	1.58±0.06[b]
Phenobarbital	23.2±1.6[b]	245±6[b]	2.77±0.18[b]	27.22±1.33[b]	2.48±0.03[b]
3-MCA	18.4±1.3	1,140±42	2.32±0.13[b]	8.18±1.17	1.26±0.03

[a] Mice received 0.8 mg·kg^{-1}·day^{-1} for 6 days. Data represent mean±SE of at least 3 sets of pooled livers from 3 mice.
[b] Values are significantly different from controls ($P<0.05$).

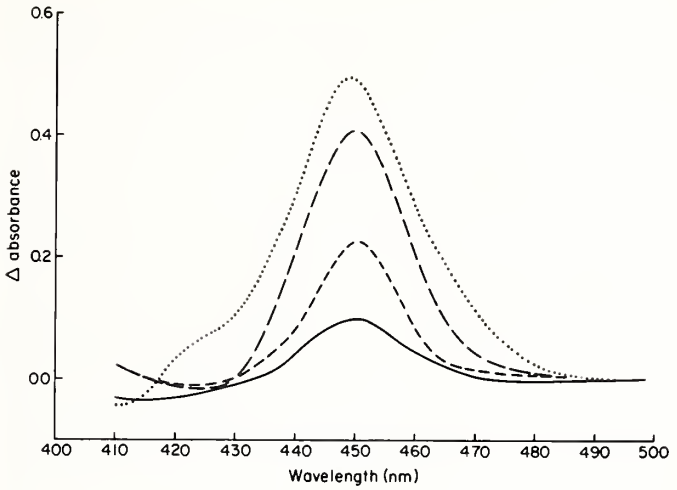

FIGURE 3.—Comparative carbon dioxide-difference spectra of rat liver microsomes in animals treated with various inducers of hepatic drug-metabolizing enzyme activity. ———— = control; ---------- = 8-MOP; – – – – – – – – – = phenobarbital; ..... = 3-MCA.

TABLE 4.—*Effect of orally administered 8-MOP to Sprague-Dawley rats on hepatic cytochrome P-450 levels and drug-metabolizing enzyme activities*[a]

Measurement	Controls	8-MOP	Percent change
Liver weight, g	5.66±0.39	5.47± 0.26	−3.4
Microsomal protein, mg/g wet weight	22.5 ±0.5	23.8 ± 0.6	+5
AHH, pmol 3-OHBP·min·mg protein	83.5 ±6.2	334.5 ±19.5[b]	+400
Aniline hydroxylase, nmol peroxidase-antiperoxidase·min·mg protein	0.80±0.05	0.73± 0.03	−8.4
Ethylmorphine *N*-demethylase, nmol formaldehyde·min·mg protein	6.53±0.25	15.22± 0.05[b]	+232
Cytochrome P-450, nmol/mg protein	0.74±0.02	1.52± 0.09[b]	+204
Ethyl isocyanide difference spectra, ratio of 455:430 peaks	0.64±0.01	0.66± 0.04	

[a] Data represent mean ± SE of 5 rats.
[b] Values are significantly different from control ($P<0.05$).

Binding Spectrum of 8-Methoxypsoralen to Control Liver Microsomes

Compounds interact with hepatic microsomal suspensions to give a type I difference spectrum, characterized by a peak at about 385 nm and a trough at about 420 nm, or a type II difference spectrum, characterized by a peak at about 430 nm and a trough at about 390 nm (*24*). The addition of 8-MOP resulted in a type I difference spectrum that is identical to that of hexabarbital (fig. 4). This finding indicates that 8-MOP interacts with liver microsomes similar to other type I compounds.

Effect of Topical Application on Aryl Hydrocarbon Hydroxylase

We made an effort to determine whether topically applied 8-MOP had any induction effect on cutaneous drug metabolism. Also, the activity of the carcinogen-metabolizing enzyme, AHH, was assessed. Whereas 8-MOP had no induction effect whatsoever, 3-MCA caused a twentyfold enhancement of the skin enzyme (table 5). We found this observation interesting because it is consistent with our data in liver that indicate 8-MOP is an inducer of the barbiturate type, which characteristically had little or no induction effect on drug-metabolizing enzyme activity in the skin. Thus our (*21*) prior observation that no detectable metabolism of TMP and 8-MOP occurs in guinea pig epidermis is confirmed.

DISCUSSION

The studies reported here indicate that 1) psoralens undergo considerable metabolic transformation in mammals, and 2) the liver is the major site of these enzymatic reactions. Orally administered psoralens are almost totally absorbed in the gastrointestinal tract and about 5–10% can be detected in the feces (*25*). Within 12 hours, more than 90% of an orally administered dose of 8-MOP can be found in the urine in the form of at least 5 metabolites that have now been partially characterized (fig. 2). These metabolites may be bound to glucuronide and hydroxylated species are also present. One of the major metabolites with a molecular weight of 232 appears to be either an epoxide or a hydroxylated derivative.

Our data indicate that oral administration of TMP to mice results in its conversion to DMCP by microsomal

TABLE 3.—*Effect of chronic oral administration of 8-MOP and TMP to hairless mice on hepatic cytochrome P-450 levels and drug-metabolizing enzyme activities*[a]

Treatment	Microsomal protein, mg/g wet tissue weight	AHH, pmol 3-OHBP·min^{-1}·mg^{-1} protein	Ethylmorphine *N*-demethylase, nmol formaldehyde·min^{-1}·mg^{-1} protein	Cytochrome P-450, nmol/mg protein
Control	12.6±1.4	124±13	13.2 ±0.8	0.78±0.03
8-MOP	13.4±0.9	122±14	2.22± 1.5[b]	1.23±0.14[b]
TMP	12.2±1.0	117±15	12.8 ±0.7	0.08±0.05

[a] Mice were given 1.2 mg·kg^{-1}·day^{-1}, 5 days/wk for 6 mo. Data represent mean ± SE of at least 3 separate sets of pooled livers from 2 mice.
[b] Values are significantly different from controls ($P<0.05$).

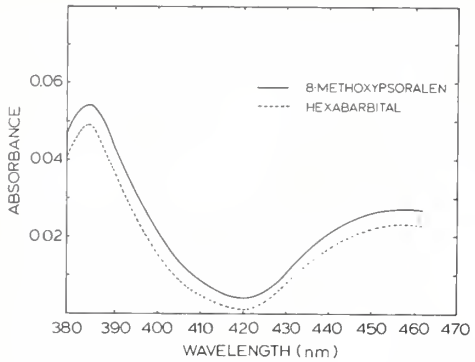

FIGURE 4.—Binding spectrum of 8-MOP to control rat liver microsomes.

enzymes in the liver; oxygen and NADPH are necessary for maximum catalytic activity. Similarly, in vitro incubation of mouse liver microsomes with TMP yields DMCP as well as several other metabolites. On the other hand, 8-MOP is appreciably metabolized in mouse liver following oral administration of the compound, whereas in vitro incubation with mouse or rat liver microsomes showed little or no evidence of metabolite formation. A major limiting factor in the in vitro assay systems appears to be the poor water solubility (4–5 mg/100 ml) of these compounds. The importance of these metabolic changes in the psoralens relates to their conversion into moieties that are much less photosensitizing and which bind poorly to DNA in vitro (*22*).

Our results show that treatment of mice and rats with orally or ip administered psoralens causes enhancement of hepatic microsomal drug metabolism and an increase in spectrally detectable cytochrome P-450. Of the 3 psoralens studied in mice, only 8-MOP had consistently significant effects on drug-metabolizing enzymes and cytochrome P-450. Thus short-term (daily for 6 days) 8-MOP administration caused significant increases in hepatic microsomal protein concentration, AHH and ethylmorphine *N*-demethylase activities, and cytochrome P-450 in mouse liver. Chronic administration (five times weekly for 6 mo) of 8-MOP also caused significant increases in ethylmorphine *N*-demethylase activity and in cytochrome P-450. Short-term administration of 8-MOP to Sprague-Dawley rats caused significant increases in hepatic microsomal AHH, ethylmorphine *N*-demethylase, and cytochrome P-450, whereas TMP had no effect on any of these measurements.

Spectral studies of the 8-MOP-induced heme protein indicated that the absorbance maximum was at 450 nm, similar to that of control or phenobarbital-treated animals. Ethyl isocyanide difference spectra also showed that microsomes from 8-MOP-treated animals demonstrated a 455:430 peak ratio similar to that of control or phenobarbital-treated animals.

Our results indicate that 8-MOP is a moderately potent inducer of hepatic microsomal drug-metabolizing enzymes and cytochrome P-450 and that the induction response to this drug is analogous though less dramatic than that which occurs in animals treated with the barbiturates.

TABLE 5.—*Comparative effect of topical application of 3-MCA and 8-MOP on cutaneous AHH in neonatal rats*

Treatment	AHH, pmol 3-OHBP·min^{-1}·mg^{-1} protein[a]
Control	0.58±0.06
3-MCA	10.36±0.17[b]
8-MOP	0.60±0.08

[a] Data represent mean ± SE of 5 experiments.
[b] Value is significantly different from control ($P<0.05$).

Application of 8-MOP to the skin of neonatal rats had no effect on cutaneous AHH, an enzyme in this tissue that normally increases in activity following exposure to polycyclic aromatic hydrocarbon carcinogens (*14*).

In summary, our data reveal that both of the psoralens most commonly used in photochemotherapy are substrates for the hepatic cytochrome P-450-dependent microsomal monooxygenase. This enzyme system appears to play a major role in the biotransformation and inactivation of these potent photosensitizing drugs. Furthermore, these agents are capable of causing moderate induction of the P-450 system, and the induction response closely parallels that seen with the barbiturate category of enzyme inducers. Thus the psoralens are another group of drugs that are acted on and in turn can influence the activity of the hepatic mixed function oxidase.

REFERENCES

(*1*) PATHAK MA, DANIELS F, FITZPATRICK TB: The presently known distribution of furocoumarins (psoralens) in plants. J Invest Dermatol 38:225–239, 1962

(*2*) MOSHER DB, PATHAK MA, FITZPATRICK TB: Vitiligo: Etiology, pathogenesis, diagnosis, treatment. *In* Update – Dermatology in General Medicine (Fitzpatrick TB, Eisen AZ, Wolff K, et al, eds). New York: McGraw-Hill, 1983, pp 205–225

(*3*) PARRISH JA, FITZPATRICK TB, TANENBAUM L, et al: Photochemotherapy of psoriasis with oral methoxypsoralen and longwave ultraviolet light. New Engl J Med 291:1207–1211, 1974

(*4*) ROENIGK HH JR: Photochemotherapy for mycosis fungoides. Arch Dermatol 113:1047–1051, 1977

(*5*) COLE RS: Psoralen monoadducts and interstrand cross-links in DNA. Biochim Biophys Acta 254:30–39, 1971

(*6*) DALL'ACQUA F, MARCIANI S, CIAVATTA L, et al: Formation of interstrand cross-linking in the photoreactions between furocoumarins and DNA. Z Naturforsch [B] 26: 561–569, 1971

(*7*) PATHAK MA, PARRISH JA, FITZPATRICK TB: Psoralens in photochemotherapy of skin disease. Farmaco [Sci] 36:479–491, 1981

(*8*) LJUNGGREN B, CARTER DM, ALBERT J, et al: Plasma levels of 8-methoxypsoralen determined by high-pressure liquid chromatography in psoriatic patients ingesting drug from two manufacturers. J Invest Dermatol 74:59–62, 1980

(*9*) CONNEY AH: Pharmacological implications of microsomal enzyme induction. Pharmacol Rev 19:317–366, 1967

(*10*) MANDULA BB, PATHAK MA, DUDEK G: Photochemotherapy: Identification of a metabolite of 4,5'8-trimethylpsoralen. Science 193:1131–1133, 1976

(*11*) PATHAK MA, KRÄMER DM: Photosensitization of skin in vivo by furocoumarins (psoralens). Biochim Biophys Acta 195:197–206, 1969

(*12*) LOWRY OH, ROSEBROUGH NJ, FARR AL, et al: Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275, 1951

(*13*) BICKERS DR, KAPPAS A, ALVARES AP: Differences in inducibility of cutaneous and hepatic drug-metabolizing enzymes and cytochrome P-450 by polychlorinated biphenyls and 1,1,1-trichloro 2,2 bis-(*p*-chlorophenyl) ethane (DDT). J Pharmacol Exp Ther 188:300–309, 1974

(*14*) MUKHTAR H, BICKERS DR: Drug metabolism in skin: Comparative activity of the mixed-function oxidases, epoxide hydratase, and glutathione-*S*-transferase in liver and skin of the neonatal rat. Drug Metab Dispos 9:311–314, 1981

(*15*) OMURA T, SATO R: The carbon monoxide binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J Biol Chem 239:2370–2378, 1964

(*16*) ALVARES AP, MANNERING GJ: Two substrate kinetics of drug metabolizing enzyme systems of hepatic microsomes. Mol Pharmacol 6:206–212, 1970

(*17*) NASH T: The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. J Biochem 55:416–421, 1953

(*18*) IMAI Y, ITO A, SATO R: Evidence of biochemically different types of vesicles in the hepatic microsomal fraction. J Biochem 60:417–428, 1966

(*19*) NEBERT DW, GELBOIN HV: Substrate-inducible aryl hydrocarbon hydroxylase in mammalian cell culture. I. Assay and properties of induced enzyme. J Biol Chem 243:5242–5249, 1968

(*20*) SCHENKMAN JB, REMMER H, ESTABROOK RW: Spectral studies of drug interaction with hepatic microsomal cytochrome. Mol Pharmacol 3:113–123, 1967

(*21*) MANDULA BB, PATHAK MA: Metabolic reactions in vitro of psoralens with liver and epidermis. Biochem Pharmacol 28:127–132, 1979

(*22*) PATHAK MA, MARCIANI S, GUIOTTO A, et al: A study on the relationship between photosensitizing and therapeutic activity of 4,5′,8-trimethylpsoralen and its major metabolite 4,5-dimethyl-5′-carboxypsoralen. J Invest Dermatol 81:533–539, 1983

(*23*) ALVARES AP, SCHILLING G, LEVIN W, et al: Studies on the induction of CO-binding pigments in liver microsomes by phenobarbital and 3-methylcholanthrene. Biochem Biophys Res Commun 29:521–526, 1967

(*24*) IMAI Y, SATO R: Substrate interaction with hydroxylase system in liver microsomes. Biochem Biophys Res Commun 22:620–626, 1966

(*25*) PATHAK MA, FITZPATRICK TB, PARRISH JA: Pharmacologic and molecular aspects of psoralen photochemotherapy. *In* Psoriasis: Proceedings of the Second International Symposium (Farber EM, Cox AJ, eds). New York: Yorke Medical Books, 1977, pp 262–271

DISCUSSION

D. M. Carter: Dr. Bickers, do you have any thoughts about why only 1 of the psoralens (8-MOP) seems to have an effect on the induction of mixed function oxidases?

D. R. Bickers: Both 8-MOP and TMP increase mixed function oxidases. One of the studies that we spent a great deal of time attempting to do was to test 8-MOP in vitro, add liver microsomes, and look for demethylation. It is a natural reaction, and Dr. Pathak has shown that in human subjects the urine contains 8-hydroxypsoralen, so you would suspect that a demethylation reaction has occurred. We tried every manipulation that we knew how to do. One of the problems that confronts one with in vitro work, of which you all are probably better aware than I, is that of substrate insolubility in water in trying to do in vitro studies with 8-MOP as a substrate. We even tried putting in a small amount of organic solvent, then evaporating it from the wall of the reaction vessel, and then adding the microsomes, etc. We could never show in vitro demethylation unequivocally, which we would like to think would occur.

I cannot answer your question, Dr. Carter, as to why the induction effect seems to be limited to 8-MOP compared with the other psoralens.

Carter: Please explain your system again. How are you using psoralens in your assay system?

Bickers: In vitro; in vitro is simply taking the substrate and taking the requisite co-factors, e.g., NADPH in air, and adding a microsomal suspension.

Carter: You do this with no light?

Bickers: No light is correct. Well, it was done in a darkened room, but no light is involved.

Carter: Is it just drug?

Bickers: It is just drug; right.

K. Kraemer: We have been discussing how psoralens may interact with other compounds. You could turn it around the other way and question whether someone who is receiving barbiturates, for instance, might respond abnormally to psoralen plus UVA.

Bickers: Yes. One thing that Dr. Pathak and I have tentatively discussed in the past would be to look and see whether patients who are getting concomitant medications show any alteration in excretion patterns of the psoralens. Obviously, that would be an indirect but nonetheless an interesting way to get at that question. You are quite right; it is possible that ingestion of other drugs could alter the rates of biotransformation of the psoralens.

P. Forbes: We have been curious about the fact that, under some circumstances, animals seem to show some adaptation to orally but not to topically administered 8-MOP. Suppose, for example, that you administered 8 to 10 doses of drug but did not administer irradiation until the last one. With topical treatment, the animals react to the last one just as if it were their first. However, when the psoralen is given orally under some circumstances, they act as if they had received little drug. I wonder whether you investigated the possibility of more than 1 drug administration and whether they react any differently?

Pathak: This experiment has been completed within the last 10 days. The drug was given by oral gavage to the mice. We checked them for 12 days, collected urine every day, and then analyzed the metabolites from day 1 with the hope that we altered the metabolism of the drug. We also hoped to find out whether we had new metabolites, unmetabolized psoralens, or more drug excreted, etc. Our TLC and high-pressure liquid chromatography patterns from days 1 to 10 are identical. We have no disturbed metabolism. Granted, it is a short period, but I do not know if these patterns will change in 3, 6, or 9 months; we have that bit of information.

Forbes: Is that qualitative or quantitative?

Pathak: It is qualitative at this stage. I would like to tell

the reasons why these studies were done. One of the major problems in studying these metabolites in the urine is the site of metabolism. We started to find out where the site was, i.e., whether it was just the liver or could it be in the kidney also. All tissues were actually examined by homogenization. In skin particularly, we also isolated microsomes and incubated them under in vitro conditions. Liver homogenates were done. Liver microsomes were done with rats and mice. For instance, TMP gets metabolized rapidly in liver microsomes to the extent that within 10 to 15 minutes we have hardly 20% of whatever substance we added.

On the other hand, 8-MOP metabolizes slowly under identical conditions. One fact we learned in all these experiments was that the skin microsome system could not yield the metabolite for which we were looking. We considered several points: Does this reflect more phototoxic reactions when you apply TMP topically to the mice and do you find more phototoxic reaction? When you give the drug orally because it is altered by metabolism, is that why it reacts less? I do not know, but this is the way I was proceeding with these experiments.

R. Brickl: I think we did some subchronic experiments in dogs and looked at plasma levels at the first administration after 3 months. There was an increase after repetitive dosing but that was not biologically significant.

You have to assume that PUVA treatment is not done daily, as normal animals are treated, but only three times a week. To find out where the site of action of metabolism is, I think the dog is an excellent animal model. You can put a catheter in the portal vein and then give an infusion and check for metabolites in plasma compared with a normal iv administration. Then you have the answer from the mechanism which is an oxidation of the furan moiety that probably takes place within the liver.

Absorption, Distribution, and Excretion of 8-Methoxypsoralen in HRA/Skh Mice[1, 2]

Indu A. Muni, F. Howard Schneider, Theodore A. Olsson III, *and* Matthew King[3, 4]

ABSTRACT—The tissue distribution and excretion of [^{3}H]8-methoxypsoralen (8-MOP), a well-accepted therapeutic agent for the treatment of psoriasis, was studied in hairless HRA/Skh female mice. Mice were given single oral doses of 6 mg of [^{3}H]8-MOP or 5-[^{14}C]8-MOP/kg in corn oil. Radiochemical analyses of tissues and excreta were accomplished by liquid scintillation counting. The 8-MOP appeared to be rapidly absorbed through the gastrointestinal tract, where the tritium levels were highest, followed by skin, blood, and liver; levels were lowest in fat (adipose tissue). In female HRA/Skh mice which had not been irradiated with UVA (320–400 nm), 84% of the carbon-14 and 58% of the tritium were recovered in the urine and feces within 24 hours of oral administration of 5-[^{14}C]8-MOP or [^{3}H]8-MOP, respectively. Animals that were exposed to UVA and received [^{3}H]8-MOP excreted approximately 12% less tritium in the urine and feces compared with the animals which received no UVA. — Natl Cancer Inst Monogr 66: 85–90, 1984.

Certain psoralens in combination with high-intensity UVA or PUVA are currently being used in experimental treatment of psoriasis (*1–3*). However, investigators who conducted several clinical studies have implied that PUVA may be associated with an increased risk of skin cancer in certain patients (*4–6*). The National Toxicology Program has initiated a program for the study of the toxicologic properties of this treatment. In this context, the agency has sponsored a study during which the biologic disposition of 4 ^{3}H-labeled psoralen compounds was examined.

Little is known about the disposition of psoralens in human or in laboratory animals. Pathak and co-workers (*7*) reported that 95% of 8-MOP administered to albino mice was absorbed through the GI tract. Over 90% of an administered dose of [^{3}H]8-MOP was recovered in the urine and feces of mice within 24 hours of administration. Fecal excretion ranged from 4 to 14% in the same period.

Busch et al. (*8*) reported that in rats which had been orally dosed with a solution of 8-[^{14}C]8-MOP, approximately 40% of the administered radioactivity was recovered in the urine and 30% in the feces. They observed the radioactivity in the feces to be secreted by way of the bile by monitoring the bile secretion after administration. About 20% of the administered radioactivity was expired through the lungs. These authors also concluded that substantial differences in absorption were observed when various pharmaceutical formulations of 8-MOP were used.

Wulf and Hart (*9, 10*) reported a qualitative description of the distribution of label after oral administration of [^{3}H]8-MOP in rats; they also found a qualitative difference in the tissue distributions in pigmented and albino mice. Subsequently, Wulf and Andreasen (*11*) determined the concentrations of [^{3}H]8-MOP, its lipid- and water-soluble metabolites, and [^{3}H]H_2O in rat tissues after oral administration of the labeled psoralen. Maximum concentrations were observed in blood, liver, and kidneys within 10 to 30 minutes after oral dosing.

We are currently investigating the pharmacokinetic and metabolic patterns of 4 [^{3}H]psoralens, i.e., 8-MOP, 5-methoxypsoralen, 3-carbethoxypsoralen, and 5-methylisopsoralen in hairless HRA/Skh mice with and without exposure of the animals to UVA. The HRA/Skh mice were selected because 1) a phototoxic response of skin after oral feeding of a psoralen and subsequent exposure to UVA radiation can be easily detected, and 2) this strain of mice is known to be susceptible to skin tumors after UVA irradiation.

Here, in addition to our description of the tissue distribution and excretion of [^{3}H]8-MOP in hairless HRA/Skh mice, is a comparison between the tissue distribution and excretion of tritium in mice which were administered [^{3}H]8-MOP and UVA and those which received the labeled psoralen only. The fecal and urinary excretion of carbon-14 after administration of 5-[^{14}C]8-MOP is also compared with the total excretion of tritium after gavage with [^{3}H]8-MOP.

MATERIALS AND METHODS

Compounds.—The 8-MOP (98.7% pure), supplied by the Midwest Research Institute (Kansas City, Mo.), was generally tritium labeled by New England Nuclear Corporation (Boston, Mass.). The specific activity of the [^{3}H]8-MOP was 1.7 Ci/mmol. The radiochemical purity, established by TLC (Silica Gel G; methylene chloride solvent system) and autoradiography, was 98.5%.

The 5-[^{14}C]8-MOP, supplied by Dr. M. A. Pathak, Massachusetts General Hospital (Boston, Mass.), had a

ABBREVIATIONS: PUVA = psoralen plus UV radiation at 320–400 nm; GI = gastrointestinal; 8-MOP = 8-methoxypsoralen; TLC = thin-layer chromatography; MPD = minimum phototoxic dose; dpm = disintegrations/minute; J = joules.

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[3] Bioassay Systems Corporation, Woburn, Massachusetts 01801.

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specific activity of 25 mCi/mmol and a radiochemical purity of at least 98% as analyzed by TLC [Silica Gel G; chloroform:ethylacetate:acetic acid (6:3:1) solvent system] and autoradiography.

Dose formulation.—We prepared separate dose formulations of 8-MOP for the tissue distribution and excretion studies by dissolving 12 mg of the unlabeled 8-MOP in 2 ml of acetone (pesticide analysis grade). The 8-MOP–acetone solutions were then dissolved in 10 ml of corn oil, and an aliquot of the [^{3}H]8-MOP (in ethanol) or [^{14}C]8-MOP (in acetone) was added to the mixture. A sufficient amount of labeled 8-MOP was added to yield a final specific activity of 10–20 μCi/mg in the dose formulation. The acetone or ethanol was then removed by rotary evaporation under reduced pressure at 37–40° C. We weighed the formulations before and after evaporation to ensure that all the acetone or ethanol was removed. Acetone-treated corn oil formulations were also prepared similarly and were used for vehicle-treated control animals.

Dose analysis.—Before dosing, we analyzed the 8-MOP corn oil formulations for 8-MOP content by measuring the absorbances of acetonitrile extracts spectrophotometrically at 254 nm. The radioactivity was measured by liquid scintillation counting of aliquots of the dose formulation in the scintillation cocktail.

Animals.—Hairless mutant mice, *Mus musculus* HRA/-Skh strain, were supplied by the Skin and Cancer Hospital, Temple University School of Medicine (Philadelphia, Pa.). These animals were 6–8 weeks of age and weighed 17–23 g. Outbred albino mice, *Mus musculus* CD-1 strain, supplied by the Charles River Breeding Laboratories (Wilmington, Mass.), were approximately 6–7 weeks old and weighed 18–22 g.

All animals received Zeigler Brothers, Inc. (Gardners, Pa.) N1H-07 feed, and water was given ad libitum. They were fasted 3 hours before dosing and fed 8 hours after dosing. The animal room was environmentally controlled at 76 ± 2° F and 50 ± 15% relative humidity.

Dose administration.—Dose formulations of 0.1 ml were administered by gavage. All animals received approximately 6 mg 8-MOP/kg with approximately 1–2 μCi of tritium or carbon-14. Control animals received an equivalent volume of acetone-treated corn oil.

Phototoxicity study.—Before initiation of the tissue distribution and excretion studies, the MPD of UVA was established for the HRA/Skh mice. The MPD is defined as the least dose of UVA radiation, expressed as joules/square centimeter, required to produce obvious skin reactions as erythema (redness) and swelling at various intervals after dosing the animals with 8-MOP. Animals were immobilized and exposed to UVA after oral administration of 6 mg 8-MOP/kg. Eight FR40T12/PUVA lamps, manufactured by GTE Sylvania (Waltham, Mass.) and supplied by Dr. M. A. Pathak, were used as the source of UVA. The UVA fluence or intensity was measured by an IL1700A Research Radiometer from International Light, Inc. (Newburyport, Mass.).

Tissue distribution studies.—These studies were conducted with 51 female HRA/Skh mice, 36 of which were divided into 12 groups of 3 each. These groups of animals were decapitated at intervals of 0.25, 0.5, 1, 1.5, 2, 4, 8, 24, 48, 72, and 96 hours after administration of 6 mg [^{3}H]8-MOP/kg. A control group was administered an acetone-treated corn oil formulation. Immediately after decapitation, trunk blood was collected in glass tubes containing EDTA. The following tissues were removed, weighed, and processed for radioactivity measurements: blood, skin, heart, lungs, liver, kidneys, brain, GI tract, spleen, muscle (thigh), adipose from inguinal region (fat), and eyes.

To investigate the effects of UVA equivalent to the MPD, we dosed 15 female HRA/Skh mice with [^{3}H]8-MOP as previously described. These 15 animals were separated into 5 groups of 3 animals/group, dosed with 6 mg [^{3}H]8-MOP/kg, and then given UVA equivalent to the MPD after 90 minutes. The animals were killed by groups at the following intervals after the UVA administration was completed: 1.5, 4, 24, and 48 hours. A control group was given acetone-treated corn oil, irradiated with UVA equivalent to the MPD value, and killed. Tissues and blood samples were collected as previously described.

Excretion studies.—The excretion study with 8-MOP was performed separately from the tissue distribution study with 25 HRA/Skh female mice, 5 male HRA/Skh mice, and 5 CD-1 female mice. The female HRA/Skh mice were divided into 4 groups: 1) 10 mice dosed with [^{3}H]8-MOP but not irradiated with UVA; 2) 5 vehicle controls dosed with acetone-treated corn oil and no UVA; 3) 5 mice administered [^{3}H]8-MOP and UVA irradiation equivalent to the MPD value; and 4) 5 vehicle control mice treated with the UVA. A group of 5 female HRA/Skh were dosed with [^{14}C]8-MOP but were not irradiated. Five male HRA/Skh mice and 5 female CD-1 albino mice were treated with [^{3}H]8-MOP and were not irradiated. The animals were individually housed in stainless steel metabolism cages obtained from Wahmann Manufacturing Company (Timonium, Md.), designed to separate feces from urine with minimum cross contamination. Each animal was dosed by gavage and immediately placed in the metabolism cage.

Due to the small amount of urine excreted by the HRA/Skh mice, a glass or plastic petri dish was placed below the fecal collection screen for uniform efficiency in the collection of the urine. At each collection interval, the feces were removed and the screen and plate were rinsed with 5–10 ml deionized water into a collection tube. The exact volumes of diluted urine were measured before processing for radioactivity measurement. All feces were dried at room temperature for 24 hours and weighed. Urine was collected at intervals of 8, 24, 48, 72, and 96 hours after psoralen administration. Feces were collected at 24, 48, 72, and 96 hours after dosing.

For those animals irradiated at the MPD level, urines were collected at 8, 24, 48, 72, and 96 hours after 8-MOP administration. Again, feces were collected at 24, 48, 72, and 96 hours after dosing.

Radiometric analyses.—All radioactivity measurements were made with a Beckman LS-100C Liquid Scintillation Counter, Beckman Instruments Inc. (Irvine, Calif.). Counts per minute were converted to disintegrations/minute by internal standardization (*12*).

We analyzed corn oil formulations by weighing 5- to 10-mg aliquots into scintillation vials, mixing with 15 ml of

Econofluor scintillation cocktail (New England Nuclear) and measuring the radioactivity. Tissue samples were prepared (*13*) for radioactivity measurements as follows: Skin, lungs, heart, kidneys, brain, spleen, muscle (thigh), fat, blood, and eyes were placed in a Model 306 TriCarb Sample Oxidizer obtained from Packard Instrument Company, Inc. (Downers Grove, Ill.). The [^{3}H]H_2O from an oxidized sample was automatically collected in Monophase-40 (New England Nuclear) scintillation cocktail and measured for radioactivity. Samples of liver and GI tract were placed in a tissue homogenizer obtained from Polytron Brinkman Instruments (West Newbury, N.Y.) with approximately 5 ml water/g tissue. Homogenate aliquots of 0.1 ml were mixed with 15 ml of Monophase-40 before the radioactivity measurements were taken. We analyzed urine samples by mixing a 0.150- or 0.250-ml aliquot of urine with 15 ml of the Monophase-40.

Fecal samples from mice treated with vehicle control and [^{3}H]8-MOP were analyzed with the use of a Tri Carb Sample Oxidizer. Samples from mice treated with [^{14}C]8-MOP or with vehicle controls were analyzed by solubilization with peroxide–perchloric acid (*14*). All fecal samples were air dried for 24 hours, pulverized, and individually mixed before sampling for analysis.

RESULTS

The results of the tissue distribution study indicate that [^{3}H]8-MOP is rapidly absorbed after oral administration. A summary of the percent distribution of tritium in various tissues for each interval when animals were killed and after dose administration is given in table 1.

The blood levels of tritium increased rapidly to a maximum of 3–4% (1- to 2-μg equivalents 8-MOP/ml blood) of the total administered dose within 15 minutes of administration, whereas the levels in the GI tract decreased to less than 50% after 30 minutes. The level of radioactivity in the skin also increased rapidly to 3–4% (1- to 2-μg equivalents 8-MOP/g tissue) of the total dose but remained at this level for 8–24 hours after administration before decreasing to about 1% after 96 hours. The blood level dropped to approximately 1% after 8 hours. Less than 3% of the administered tritium remained in the body after 96 hours.

The GI tract always showed the highest radioactivity followed by the skin, blood, liver, and muscle. The accumulation of tritium in the fat tissue was insignificant at all times. When the tissue distribution data were expressed in relative activity with respect to the whole body (table 2), the distribution pattern in each organ remained the same. The GI tract, blood, kidneys, liver, and skin maintained the higher levels of relative activity. As illustrated in figure 1, this is also true when the distribution of radioactivity was expressed as microgram equivalents of 8-MOP/gram tissue.

The results of the excretion study, summarized in table 3, indicate that most of the radioactivity as tritium or carbon-14 was excreted in the urine within the first 24 hours after administration. Among male or female

TABLE 1.—*Percent distribution (recovery) of radioactivity of tritium per tissue compared with the total radioactivity administered as 6 mg [^{3}H]8-MOP/kg in corn oil*[a]

Time after dose administration, hr	Skin	Heart	Lungs	Kidneys	Brain	Spleen	Muscle	Fat	Liver	GI tract	Blood	Total recovered
No UVA												
0.25	2.8	0.1	0.1	0.9	0.2	0.0	1.6	0.2	2.0	55.1	3.6	67.3
0.50	2.3	0.1	0.1	0.5	0.1	0.1	1.0	0.2	2.1	47.9	1.5[b]	55.1
1.00	2.8	0.1	0.2	0.7	0.3	0.1	2.9	0.3	3.4	31.8	3.0	45.4
1.50	3.3	0.1	0.1	0.4	0.4	0.1	2.3[b]	0.2	3.8	24.2	3.3[b]	37.1
2.00	3.8	0.1	0.2	0.3	0.3	0.1	2.9	0.1	2.9	30.0	3.0	43.6
4.00	3.5	0.1	0.2	0.3	0.5	0.2	3.0	0.1	2.3	13.9	3.1	26.9
8.00	3.7	0.1	0.1	0.2	0.4	0.0	0.8	0.0	2.1	11.4	1.2	20.1
24.00	2.6	0.1	0.1	0.2	0.2	0.1	1.8	0.0	1.3	2.8	0.6	9.7
48.00	1.6	0.0	0.1	0.1	0.2	0.0	1.5	0.1	0.9	1.8	0.7	6.9
72.00	1.1	0.0	0.1	0.1	0.1	0.0	1.0	0.0	0.5	1.1	0.4	4.5
96.00	0.8	0.0	0.0	0.1	0.1	0.0	0.6	0.1	0.3	0.6	0.3	2.7
UVA												
3.50	3.5	0.1	0.1	0.2	0.3	0.1	2.3	0.1	3.7	15.1	1.4	26.8
6.00	3.8	0.1	0.2	0.3	0.4	0.1	3.4	0.1	1.9	10.0	2.4	22.7
26.00	1.7	0.1	0.1	0.2	0.2	0.0	2.0	0.1	1.2	2.5	0.3	8.3
50.00	1.5	0.0	0.1	0.1	0.1	0.0	1.9[b]	0.1	0.7	1.7	1.5	7.1

[a] Total radioactivity administered to each animal was 4.8×10^6 dpm [^{3}H]8-MOP (2.2 μCi). All results represent the mean values of animals/group. These values are the percent radioactivity recovered in the entire tissue. The total weights of skin, muscle, fat, and blood were calculated from the following percent weight factors (tissue weight to total body weight): skin, 15%; muscle, 19%; fat, 3.2%; and blood, 8%. No UVA indicates the animals in this group were not treated with UV radiation; UVA indicates the animals in this group were treated with the MPD value of UV radiation which was 30 min of UVA exposure at 3–9 J/cm^2 skin surface area, 90 min after [^{3}H]8-MOP administration. No recovery was noted in eyes. No. of animals/group = 3, except the 96-hr group, which had 2.

[b] Values are for 2 animals.

TABLE 2.—*Relative activity of tritium in each tissue with respect to the total radioactivity administered in each animal*[a]

Time after dose administration, hr	Skin	Heart	Lungs	Kidneys	Brain	Spleen	Muscle	Fat	Eyes	Liver	GI tract	Blood
No UVA												
0.25	18.8	20.8	18.8	68.9	8.3	10.5	8.6	5.0	5.6	41.5	365.2	44.9
0.50	15.7	9.5	13.0	35.9	7.6	11.6	5.5	4.7	4.4	36.0	355.8	32.9[b]
1.00	18.9	17.9	24.8	47.1	16.7	20.7	15.4	8.5	8.8	58.8	245.3	37.3
1.50	21.7	14.6	16.8	46.1	18.4	17.8	12.3[b]	4.7	5.2	61.8	182.8	41.2[b]
2.00	25.1	16.9	21.6	29.7	18.0	16.7	15.3	3.7	5.6	49.0	214.9	36.9
4.00	23.2	19.4	20.9	23.3	22.7	19.2	15.6	3.0	10.8	36.5	124.6	38.5
8.00	24.6	12.1	14.6	18.5	19.6	6.5	4.0	2.2	1.6	36.0	103.2	16.5
24.00	15.9	12.4	12.7	13.3	12.3	10.6	7.3	1.5	2.4	20.3	20.8	7.2
48.00	10.9	8.5	9.3	9.8	8.5	8.7	7.8	2.2	4.7	13.1	10.4	8.3
72.00	7.2	6.8	6.7	7.0	6.6	6.1	5.4	1.4	3.4	7.5	7.9	4.7
96.00	4.9	4.3	4.4	4.3	4.0	2.0	3.0	2.0	2.0	3.7	4.4	3.2
UVA												
3.50	21.7	16.6	15.3	17.3	13.4	12.0	12.1	2.5	5.4	51.3	132.7	17.9
6.00	25.6	20.0	21.3	23.6	21.4	20.4	17.8	3.2	10.3	30.8	82.5	29.4
26.00	11.4	11.5	12.0	11.6	10.5	7.2	10.5	1.6	3.9	17.8	17.5	3.2
50.00	9.8	7.4	7.9	8.4	7.7	6.9	10.2[b]	2.7	6.2	10.7	11.2	18.7

[a] The relative activity was expressed as: (microcuries activity recovered/tissue weight in grams)/(total activity administered/total body weight) × 100. The total activity administered was 2.2 μCi or 6 mg [^{3}H]8-MOP/kg in corn oil. All of the above results are mean values of the sample size. No UVA indicates the animals in this group were not treated with UV radiation but were administered [^{3}H]8-MOP; UVA indicates the animals in this group were treated with the MPD value of UV radiation, which was 30 min of UVA exposure at 3–9 J/cm^2 of skin surface area, 90 min after the oral administration of the psoralen. No. of animals/group = 3, except the 96-hr group, which had 2.

[b] Values are for 2 animals.

HRA/Skh or female CD-1 albino mice, little difference in the excretion of tritium radioactivity was noted. The female HRA/Skh mice which were dosed with 5-[^{14}C]8-MOP excreted about 54% of the administered dose in the urine and 30% of the dose in the feces within 24 hours of dose administration. Less than 2.0% of the carbon-14 was excreted in the feces and less than 1% was excreted in the urine between 24 and 96 hours after dose administration.

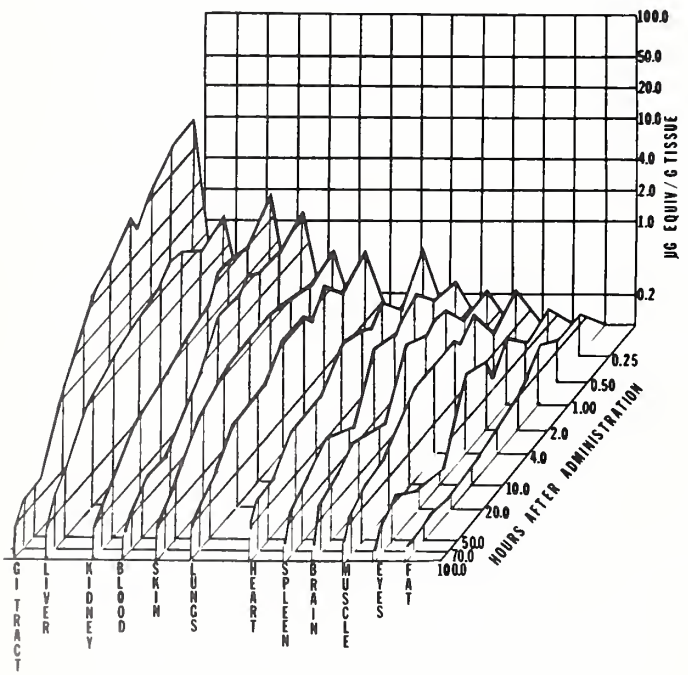

FIGURE 1.—Tissue distribution of [^{3}H]8-MOP expressed as microgram equivalents of 8-MOP/gram of tissue.

Female HRA/Skh mice which were administered [^{3}H]8-MOP excreted 46% of the label in the urine and 11% in the feces within 24 hours. Less than 1% was excreted in the urine or feces between 24 and 96 hours after dose administration. The phototoxicity study established the MPD as 30 minutes of UVA exposure, at 3–9 J/cm^2 of skin surface area, irradiated 90 minutes after 6 mg 8-MOP/kg by corn oil gavage.

When compared with non-UVA-exposed animals, approximately 16% less tritium was recovered in the urine of irradiated mice, whereas 4% more was recovered in their feces. The irradiation of the hairless mice resulted in a total of 12% less excretion of this label in 4 days.

DISCUSSION

The rate of absorption of orally administered psoralen was rapid, as indicated by the equally rapid initial disappearance of tritium from the GI tract. With the exception of the intestines, the skin, blood, liver, and kidneys were the major organs in which most of the activity was detected. Peak levels of radioactivity were measured within 4 hours of administration in most tissues. Less than 3% of the label administered was recovered in the tissues at the end of the 96-hour period.

Urinary excretion was the major route of elimination of [^{3}H]8-MOP and [^{14}C]8-MOP in hairless HRA/Skh mice in the 96-hour excretion study. Within 24 hours of adminis-

TABLE 3.—*Urinary and fecal excretion of tritium or carbon-14 after the oral administration of 6 mg [^{3}H]8-MOP or 5-[^{14}C]8-MOP/kg in corn oil*[a]

Treatment	Dose group		Urinary excretion					Fecal excretion				Total excreted
	No. of mice/ group	Sex	Hours after administration: 8	24	48	48–96	Urinary total	Hours after administration 24	48	48–96	Fecal total	
[^{3}H]8-MOP, no UVA[b]	10	Female	43.2	2.9	0.5	<0.1	46.6	11.2	0.6	<0.5	11.7	58.3
[^{3}H]8-MOP, UVA	5	"	28.3	1.9	0.4	<0.1	30.6	15.0	0.5	<0.5	15.5	46.1
[^{14}C]8-MOP, no UVA	10	"	51.0	3.3	0.6	<0.1	54.9	28.0	2.0	<0.5	30.0	84.9
[^{3}H]8-MOP, no UVA	5	Male	45.8	1.5	0.3	<0.1	47.6	14.1	0.7	<0.5	14.8	62.4
[^{3}H]8-MOP, no UVA	5	Female	42.3	2.2	0.1	<0.1	44.6	9.1	0.5	<0.5	9.6	54.2

[a] All results are mean values expressed as percent radioactivity recovered compared with the total radioactivity administered. The total radioactivity administered was 4.88×10^6 dpm [^{3}H]8-MOP (2.2 μCi) or 4.96×10^6 dpm 5-[^{14}C]8-MOP (2.3 μCi). No UVA indicates the animals of this group were not irradiated; UVA indicates the animals of this group were treated with MPD value of UV radiation which was 30 min of UVA exposure at 3–9 J/cm^2 skin surface area, 90 min after [^{3}H]8-MOP administration.

[b] HRA/Skh mice were used for first 4 treatment groups, CD-1 in the fifth.

tration of [^{3}H]8-MOP in corn oil, 46–47% of the label was recovered in the urine of male or female HRA/Skh mice and 12–15% was recovered in the feces. The total tritium radioactivity excreted in these mice was 58–62% of the administered dose. Urinary excretion of carbon-14 in female HRA/Skh mice represented about 54% of that administered, and 30% of the activity was eliminated in the feces. The total excretion in the urine and feces combined was 84% of the administered dose in a 4-day period. Most of the radioactivity as carbon-14 (54%) and tritium (47%) was excreted in the first 24 hours and less than 1% was excreted during the remaining 3 days.

Thirty percent of the administered radioactivity was measured in the feces of mice treated with 5-[^{14}C]8-MOP compared with 10–15% recovered in the mice treated with [^{3}H]8-MOP. This may be explained by the fact that a considerable amount of the latter label may have exchanged with endogenous water. In addition, our recent unpublished observations suggest that significant levels of [^{3}H]H_2O may have been excreted through the skin and lungs. Also, Busch and co-workers (*8*) reported biliary excretion of 8-MOP into the intestine in rats. This route of excretion may account for the significant fecal excretion of both labels in this study for mice treated with radiolabeled 8-MOP.

Minor variations in the total excretion of tritium were observed between animals which received the PUVA treatment and those administered psoralen only. The total urinary and fecal excretion of tritium was 12% less for the UVA-treated mice; this decreased excretion could be due to the increased loss of water from skin or to the photoconjugation of [^{3}H]8-MOP to skin proteins and DNA, or both. The photoconjugation hypothesis appears to be less probable because the skin levels of tritium for the UVA-treated and untreated animals were similar (*see* table 1). The tissue distribution data indicated little or no significant difference in the distribution of radioactivity in mice which were or were not treated with PUVA.

The pooled urine samples from various test groups will be subjected to high-performance liquid chromatographic and TLC analyses for the metabolic patterns of 8-MOP. The results of these analyses will be reported in a future publication.

REFERENCES

(*1*) PARRISH JA, FITZPATRICK TB, TANENBAUM L, et al: Photochemotherapy of psoriasis with oral methoxsalen and longwave ultraviolet light. N Engl J Med 291:1207–1211, 1974

(*2*) WOLFF KW, FITZPATRICK TB, PARRISH JA, et al: Photochemotherapy for psoriasis with orally administered methoxsalen. Arch Dermatol 112:943–950, 1976

(*3*) PATHAK MA, KRÄMER DM, FITZPATRICK TB: Photobiology and photochemistry of furocoumarins. *In* Sunlight and Man: Normal and Abnormal Photobiologic Responses (Fitzpatrick TB, Pathak MA, Harber LC, et al, eds). Tokyo: Univ Tokyo Press, 1974, pp 335–368

(*4*) STERN RS, THIBODEAU LA, KLEINERMAN RA, et al: Risk of cutaneous carcinoma in patients treated with oral methoxsalen photochemotherapy for psoriasis. N Engl J Med 300:809–813, 1979

(*5*) URBACH F: Modification of ultraviolet carcinogenesis by photoactive agents: Preliminary report. J Invest Dermatol 32:373–378, 1959

(*6*) PATHAK MA, DANIELS F, HOPKINS CE, et al: Ultra-violet carcinogenesis in albino and pigmented mice receiving furocoumarins: Psoralen and 8-methoxypsoralen. Nature 183:728–730, 1959

(*7*) PATHAK MA, FITZPATRICK TB, PARRISH JA: Pharmacologic and molecular aspects of psoralen photochemotherapy. *In* Proceedings of the Second International Symposium on Psoriasis (Farber EM, Cox JA, eds). New York: Yorke Medical Books, 1977, pp 262–271

(*8*) BUSCH U, SCHMID J, KOSS W, et al: Pharmacokinetics and metabolite pattern of 8-methoxypsoralen in man following oral administration as compared to the pharmacokinetics in rat and dog. Arch Dermatol Res 262:255–265, 1978

(*9*) WULF HC, HART J: Accumulation of 8-methoxy-psoralen in the rat retina. Acta Ophthalmol 56:284–289, 1978

(*10*) ———: Distribution of tritium labelled 8-methoxypsoralen in the rat studied by whole body autoradiography. Acta Derm Venerol (Stockh) 59:97–103, 1979

(*11*) WULF HC, ANDREASEN MP: Distribution of ^{3}H-8-MOP and its metabolites in rat organs after a single oral administration. J Invest Dermatol 76:252–258, 1981

(*12*) LONG EC: Liquid Scintillation Counting Theory and Techniques 1976. Tech Rep No. 915-NUC-76-7T P/N 566671. Fullerton, Calif.: Beckman Instruments Inc., 1976

(*13*) Manual 2130/1, Packard Model B306 Tri-Carb Sample Oxidizer, 1977. Downers Grove, Ill.: Packard Instrument Co., Inc., 1977

(*14*) New England Nuclear: Procedure for solubilizing difficult biological material, bone and feces. *In* Liquid Scintillation Counting. Boston: New England Nuclear, 1977, p 7

Subchronic Toxicity in Rats Administered Oral 8-Methoxypsoralen[1, 2]

June K. Dunnick,[3] William E. Davis, Jr.,[4] Theodore A. Jorgenson,[4] Victor J. Rosen,[4, 5] *and* Ernest E. McConnell[3]

ABSTRACT—Scientists at the National Toxicology Program are studying the toxicologic properties of 8-methoxypsoralen (8-MOP) with and without 320–400 nm UV (UVA). The combination of psoralen and UVA is a promising treatment for psoriasis. In this study, 8-MOP was administered to male and female Fischer 344 rats without subsequent UVA exposure for the determination of toxic effects of the psoralen alone. The drug (in corn oil) was administered by gavage 5 days/week for 90 days at doses of 0, 25, 50, 100, 200, and 400 mg/kg. The effects of toxicity were seen primarily in the 200- and 400-mg/kg dose groups, which included mortality, decreased body weight gain, and dose-related increases in liver:body ratios. On histopathology, target organ toxicity was seen in the liver, testes, and adrenals. In this study, relatively high doses of 8-MOP were tolerated in comparison to the dose of psoralen used in combination therapy clinically. — Natl Cancer Inst Monogr 66: 91–95, 1984.

Scientists at the National Toxicology Program are investigating the toxic properties of orally administered 8-MOP because of recommendations from the National Cancer Institute and the Food and Drug Administration that we study the toxic properties of PUVA therapy, a term applied to the use of psoralen and UVA for the treatment of various skin diseases. In the United States, oral administration of 8-MOP in combination with UVA is used for the treatment of vitiligo and psoriasis (*1*).

Forbes and Davies (*2*) found that the route of administration of 8-MOP is important in determining the toxicity of PUVA treatment. In this study, the 8-MOP was administered by gavage to Fischer 344 rats for 13 weeks without exposure to UVA. At the end of the experiment, the animals were necropsied and examined histopathologically.

Several studies on the effects of oral administration of 8-MOP have been performed (*3–8*), but they were limited because 8-MOP was used at concentrations below the levels of toxicity demonstrated by the drug alone. Focus was primarily on the effects of 8-MOP in combination with UVA. A study of the toxic effects of orally administered 8-MOP without UVA is important in helping us to distinguish the toxic properties of the psoralen alone from those of the drug and UVA.

MATERIALS AND METHODS

Animals.—Male and female Fischer 344 rats, obtained from the Charles River Breeding Laboratory (Portage, Maine), averaged 29 days of age (± 7 days) upon receipt. The animals were quarantined for 13 days and then at a mean age of 42 ± 7 days were randomly placed into 6 dose groups by sex (control, 25, 50, 100, 200, and 400 mg/kg), 10 animals/group. Deionized water and the NIH-07 open formula diet from Zeigler Brothers, Inc. (Gardners, Pa.) were provided ad libitum. The rats, observed daily for morbidity and clinical signs of toxicity, were individually weighed once each week. They were fasted overnight before the terminal kill.

Animal housing conditions.—The animals were housed 5/cage in polycarbonate cages containing hardwood chip bedding, both of which were obtained from Lab Products, Inc. (Rochelle Park, N.J.). The shelves supporting the cages were fitted with filter sheets; cages were changed twice a week.

Yellow fluorescent F40G0 bulbs from General Electric Company (Cleveland, Ohio) provided a light–dark cycle of 12 hours each. These lamps have an output of 500–700 nm, with a maximum output at 580 nm and less than 1% output in the UV wavelengths. The cages were rotated by shelf, with those cages on the lowest shelf moved to the top shelf on a weekly schedule so that equal exposure to light was assured. Thirteen complete room exchanges of fresh air were supplied/hour. Room temperature ranged from 74 to 78° F, and relative humidity ranged from 46 to 76%.

Chemical.—The 8-MOP was obtained from Elder Pharmaceuticals (Bryan, Ohio). The compound was identified and analyzed by elemental analysis, thin-layer chromatography, high-pressure liquid chromatography, melting point, and infrared and UV light spectra at the Midwest Research Laboratories (Kansas City, Mo.) and SRI International (Menlo Park, Calif.). By gas chromatography, 8-MOP was 99.6% pure.

The chemical was dissolved in acetone and then diluted in corn oil; the acetone was subsequently removed by rotary evaporation at 40° C. Fresh batches were prepared

ABBREVIATIONS: 8-MOP = 8-methoxypsoralen; PUVA = psoralen plus UV radiation at 320–400 nm.

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[2] Supported by Public Health Service contract N01-ES38069 with the National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina.

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TABLE 1.—*Effect of 8-MOP on weight gain and survival in Fischer 344 rats*

Dose of 8-MOP, mg/kg	Average weight at wk 0 ± SD, g		Average weight at wk 13 ± SD, g		Weight gain at 13 wk[a]	
	Male	Female	Male	Female	Male	Female
Control	176±17	132±8	352±27	210±18	—	—
25	175±11	135±7	370±22	210±10	11	−4
50	175±19	131±8	361±16	205± 8	6	−5
100	172±16	134±7	311±15	213±11	−21	1
200	176±16	135±7	275±31	179±22	−44	−44
400[b]	171±15	132±8	194±28	136± 8	−87	−95

[a] Percent weight change relative to controls = $\frac{\text{weight change (dosed group)} - \text{weight change (control group)}}{\text{weight change (control group)}} \times 100$.

[b] In the groups receiving 400 mg, 6 males and 8 females died before 13 wk; all other animals survived to the end of the treatment period. Rats were fasted overnight before being killed. Ten rats were in each group.

weekly. The 8-MOP–corn oil suspension was given by gavage 5 days/week (Monday–Friday) for 13 weeks. The volume administered remained at 5 ml/kg, whereas the concentration of chemical was altered, so that the groups received daily doses of 0, 25, 50, 100, 200, and 400 mg 8-MOP/kg for 6 weeks, after which the concentration was doubled so that the volume was reduced to 2.5 ml/kg in all except the 400-mg/kg group. Analysis of these weekly batches showed that the concentrations of 8-MOP were within ± 10% of the targeted concentrations.

Pathology.—The animals were killed within 2 days after the last dosing day by ip injection of 1 ml sodium pentabarbital. Complete necropsy examinations were performed on all rats, including those that died during the exposure. Hematoxylin and eosin-stained sections of the following tissues from the 2 highest dose groups were examined microscopically: gross lesions and tissue masses (regional lymph nodes, when possible); mandibular lymph node; salivary gland; sternebrae, including marrow; thyroid; parathyroids; esophagus; stomach; small intestine; colon; liver; prostate; testes; seminal vesicles; ovaries; mammary gland; lung and trachea; heart; uterus; brain; eyes; thymus; pancreas; spleen; kidneys; urinary bladder; adrenals; pituitary; and skin. In addition, the livers of the animals receiving the 3 lower doses were examined. Liver and body weights were determined on all rats killed at the end of the study.

TABLE 2.—*Liver:body weight ratios at terminal kill in rats surviving to 13 wk*[a]

Dose of 8-MOP, mg/kg	Average liver weight ± SD, g	Average body weight ± SD, g	Liver:body weight ratios ± SD
Male			
Control	11.74± 1.67	345± 26	0.034± 0.0033
25	13.97± 1.12[b]	364± 18	0.038± 0.0029[c]
50	15.01± 1.66[b]	351± 13	0.043± 0.0043[b]
100	14.88± 1.91[b]	311± 14[b]	0.048± 0.0047[b]
200	16.44± 1.33[b]	272± 28[b]	0.061± 0.0049[b]
400	15.20± 1.69[c]	188± 37[b]	0.082± 0.0125[b]
Female			
Control	6.72± 1.02	206± 16	0.033± 0.0035
25	7.24± 0.54	203± 10	0.036± 0.0023[b]
50	7.72± 0.76	202± 7	0.038± 0.0032[b]
100	9.74± 0.68[b]	210± 10	0.046± 0.0025[b]
200	11.32± 0.93[b]	176± 19[b]	0.065± 0.0048[b]
400	12.89± 2.45[c]	130± 18[c]	0.099± 0.0054[b]

[a] See footnote *b*, table 1.

[b] $P<0.01$ vs. controls by the Mann-Whitney U test.

[c] $P<0.05$ vs. controls by the Mann-Whitney U test.

RESULTS

Survival and Clinical Signs

Toxic signs observed during the test period included depressed weight gain, increased liver weights when compared with control animals, and mortality. In the 400-mg/kg

TABLE 3.—*Histopathologic findings in Fischer 344 rats receiving 8-MOP for 13 wk*

Organ	Histopathologic finding	Sex	Dose of 8-MOP, mg/kg	
			200	400
Liver	Minimum to mild fatty change	Male	6/10	8/10
		Female	8/10	9/10
Adrenals	" " " " "	Male	0/10	7/10
		Female	0/10	7/10
Male reproductive system				
Testes, seminiferous tubules	Atrophy		1/10	4/10
Seminal vesicles	"		0/10	8/10
Prostate	"		0/10	3/10

dose group, 6 of 10 male rats died (4 during wk 2, 1 each in wk 8 and 9) and 8 of 10 female rats died (2 during wk 1 and 6 during wk 2). The chemical caused significant dose-dependent depression in body weight in the 2 highest groups in both sexes (table 1). A dose-related increase was observed in the liver:body weight ratios in both male and female rats (table 2).

Gross Pathology

At necropsy, the livers appeared enlarged and mottled in 1 male and 2 female rats in the 200-mg groups and 6 males and 5 females in the 400-mg/kg groups. Other findings at necropsy included enlarged adrenals in 3 male and 5 female rats given 400 mg/kg; abnormally small testes or seminal vesicles were noted in 3 males in this group.

Histopathology

Histopathologic examination of the affected livers revealed mild accumulations of lipid in hepatocytes, particularly in midzonal areas (table 3, fig. 1) at the 200- and 400-mg/kg dose levels. Single cell necroses of scattered hepatocytes were observed in some rats that died. Also observed at the 400-mg/kg dose level were fatty changes in the zona fasciculata and the zona reticulata of the adrenal cortex (fig. 2), and atrophy of the prostate, seminal vesicles, and seminiferous components of the testes (fig. 3).

DISCUSSION

In the United States, 8-MOP is given orally followed by exposure to UVA for the treatment of vitiligo and psoriasis. In our studies, it was given by gavage to Fischer 344 rats so that the toxic properties of psoralen alone could be determined.

Target organ toxicity was seen at the highest doses (200 and 400 mg/kg) manifested by mild fatty changes in the liver and adrenal cortex and by atrophy of male genital organs. These fatty changes were not believed to be life-threatening. Elevated liver:body weight ratios were also seen in dosed animals. Using radiolabeled 8-MOP, Wulf and Andreasen (*9*) performed distribution studies in the rat and have shown that the psoralen, particularly water-soluble metabolites, concentrates in the liver. The psoralen is also transported to the adrenals in low concentrations. Thus the target organ toxicity of 8-MOP in the liver and adrenals correlated with its distribution to these organs. Because 8-MOP has been shown to induce liver microsomal mixed function oxidases, some investigators (*10, 11*) believe that these enzymes are involved in its metabolism. The atrophy of the male genital organs occurred in the 400-mg/kg group, a group that exhibited severe weight loss. Determination of whether this atrophy was due to debilitation or some other cause, such as an endocrinologic effect, requires additional research.

At the high doses, 8-MOP caused decreased body weight gains (200 mg/kg) and death (400 mg/kg) in male and female rats. Other workers (*12*) reported that 50% of their animals died when 791 mg 8-MOP/kg were given orally. The form of the drug, solubility, and frequency of administration affect the rate of absorption and the median lethal dose results.

The average human dose of 8-MOP used in clinical studies in combination with UVA light is less than 1 mg/kg

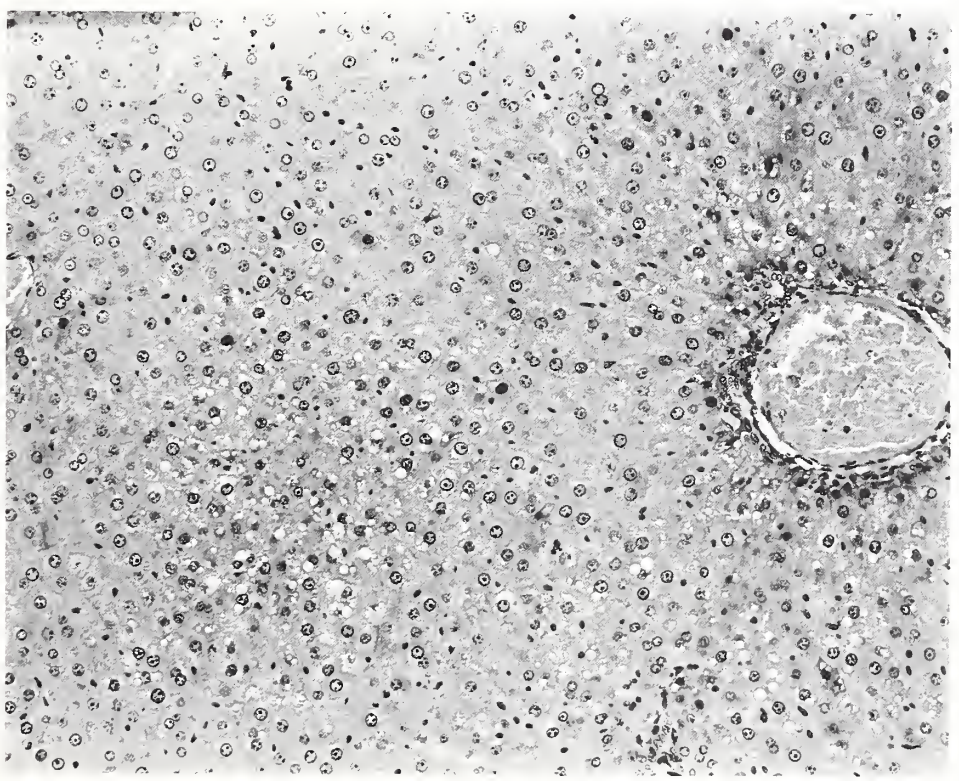

FIGURE 1.—Liver of a male Fischer 344 rat which received 200 ppm 8-MOP showing mild accumulation of fat in the midzonal area. Central vein on *left*. Portal tract in *top right corner*. × 160

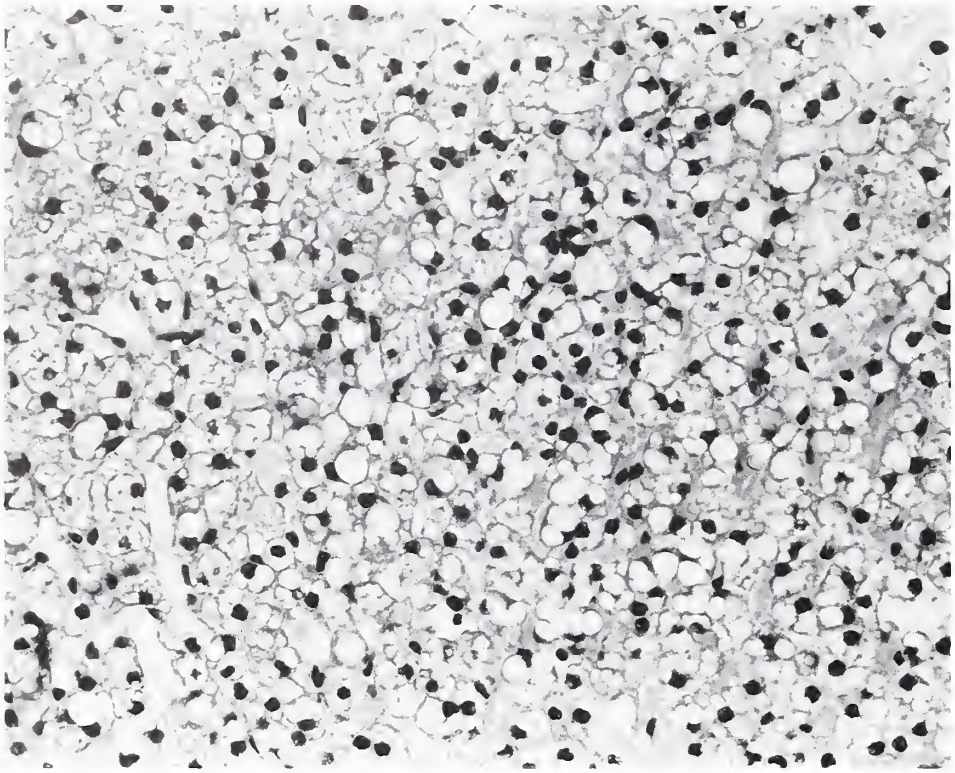

FIGURE 2.—Adrenal cortex of a male Fischer 344 rat which received 400 ppm 8-MOP showing mild accumulation of fat in the zona fasciculata. × 430

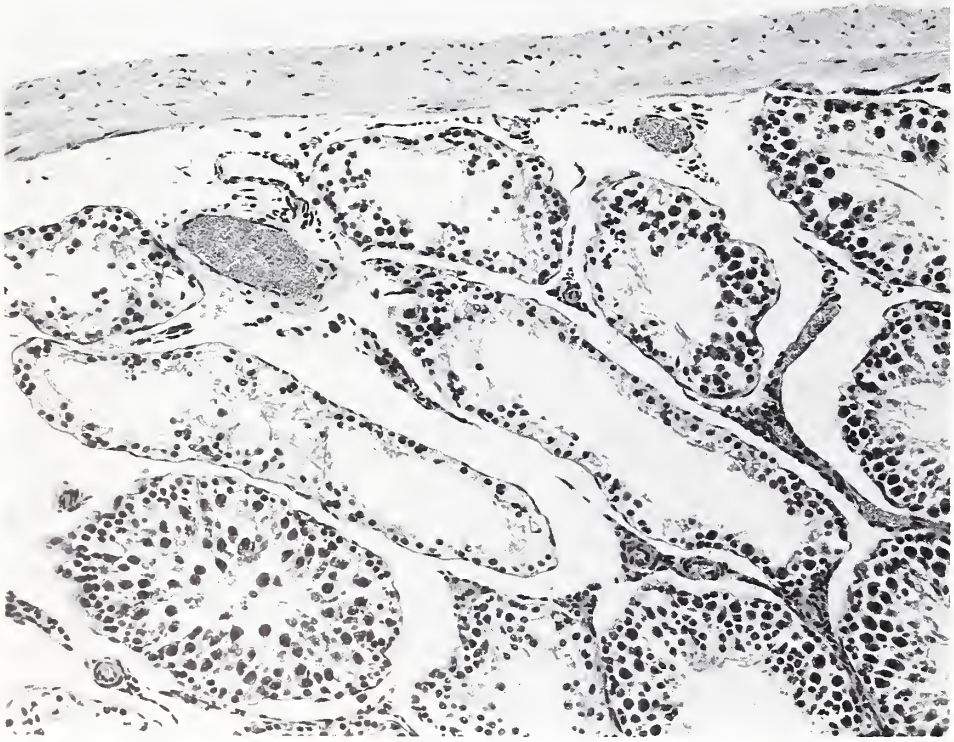

FIGURE 3.—Testicle of a Fischer 344 rat which received 200 ppm of 8-MOP showing focal seminiferous tubular atrophy in a few tubules, particularly at the *top*. × 160

(7). Exact correlations between doses in rodents and humans cannot be made. However, when we gave 8-MOP without subsequent UVA light exposure, toxicity was found only at doses 100 or more times the dose used in humans when compared on a milligram/kilogram basis. If the toxic dose of psoralen in rats is estimated at 200 mg/kg and the human dose is 0.5 mg/kg, the toxic dose in rats is 60 times more than the human dose of 1 mg/cm^2.

These studies on the subchronic effects of 8-MOP indicate that it is toxic for several tissues including the liver; alterations in the liver:body weight ratios were seen in all dosed groups.

REFERENCES

(1) PARRISH JA, FITZPATRICK TB, TANENBAUM L, et al: Photochemotherapy of psoriasis with oral methoxsalen and longwave ultraviolet light. N Engl J Med 291:1207–1211, 1974

(2) FORBES PD, DAVIES RE: Enhancement of photocarcinogenesis by 8-MOP. *In* Psoralens in Cosmetics and Dermatology (Cahn G, Forlot BP, Grupper C, et al, eds). New York: Pergamon Press, 1981, pp 365–370

(3) GRIFFIN AC, HAKIM RE, KNOX J: The wavelength effect upon erythemal and carcinogenic response in psoralen-treated mice. J Invest Dermatol 31:289–295, 1958

(4) GRIFFIN AC: Methoxsalen in ultraviolet carcinogenesis in the mouse. J Invest Dermatol 32:367–372, 1959

(5) URBACH F: Modification of ultraviolet carcinogenesis by photoactive agents. J Invest Dermatol 32:373–378, 1959

(6) PATHAK MA, DANIELS F JR, HOPKINS CE, et al: Ultraviolet carcinogenesis in albino and pigmented mice receiving furocoumarins: Psoralen and 8-methoxypsoralen. Nature 183:728–730, 1959

(7) HAKIM RE, GRIFFIN AC, KNOX JM: Erythema and tumor formation in methoxsalen-treated mice exposed to fluorescent light. Arch Dermatol 82:572–577, 1960

(8) LANGNER A, WOLSKA H, MARZULLI FN, et al: Dermal toxicity of 8-methoxypsoralen administered (by gavage) to hairless mice irradiated with long-wave ultraviolet light. J Invest Dermatol 69:451–457, 1977

(9) WULF HC, ANDREASEN MP: Distribution of ^{3}H-8-MOP and its metabolites in rat organs after a single oral administration. J Invest Dermatol 76:252–258, 1981

(10) MANDULA BB, PATHAK MA, NAKAYAMA Y, et al: Induction of mixed-function oxidases in mouse liver by psoralens. Br J Dermatol 99:687–692, 1978

(11) TSAMBAOS D, VIZETHUM W, GOERZ G: Effect of oral 8-methoxypsoralen on rat liver microsomal cytochrome P-450. Arch Dermatol Res 263:339–342, 1978

(12) APOSTOLOU A, WILLIAMS RE, COMERESK CR: Acute toxicity of micronized 8-methoxypsoralen in rodents. Drug Chem Toxicol 2:309–313, 1979

Difference in Topical and Systemic Reactivity of Psoralens: Determinations of Epidermal and Serum Levels[1, 2]

Andrija Kornhauser,[3, 4] Wayne G. Wamer,[3] *and* Albert L. Giles, Jr.[3]

ABSTRACT—Topical application of 8- and 5-methoxypsoralen (8-MOP, 5-MOP), psoralen, and 4,5′,8-trimethylpsoralen (TMP) to the epilated backs of albino guinea pigs followed by UV irradiation at 320–400 nm resulted in elicitation of similar phototoxicity. However, the systemic phototoxicity determined after oral administration of these psoralen derivatives differed significantly. Serum and epidermal levels of 8-MOP, 5-MOP, and TMP were determined 2 hours post administration. We found a linear relationship between serum and epidermal levels of 8-MOP, but the levels of the other psoralen derivatives were significantly lower. We believe that the reduced oral phototoxicity of 5-MOP and TMP relative to 8-MOP in the guinea pig is due to its reduced levels in the epidermis. — Natl Cancer Inst Monogr 66: 97–101, 1984.

Psoralens are a class of natural products present in many plants including parsley, parsnips, celery, and citrus fruits. These naturally occurring psoralens, such as 8-MOP, 5-MOP, and TMP (fig. 1), have been identified as phytoalexins; they are part of the plant's defensive response against fungal and insect challenges (*1–3*). In addition, psoralens have long been known as powerful phototoxic agents in man and animals.

Human exposure to psoralens is becoming increasingly frequent: topically through contact with psoralen-containing fruits and vegetables (*4*) or cosmetics (*5, 6*), and orally through fruits and vegetables common in our diets (*7*). Ivie et al. (*7*) have estimated that 100 g of parsnips would expose an individual to 4–5 mg of total psoralens. The consequences of repeated exposures at these levels are not fully understood. The use of psoralen-containing drug formulations, particularly in photochemotherapy, is also an important source of oral exposure to psoralens. Photochemotherapy with 8-MOP and UVA is rapidly becoming established as a treatment for skin diseases such as vitiligo (*8*), mycosis fungoides (*9*), and, particularly, psoriasis (*10*). Recently, the Food and Drug Administration approved the use of 8-MOP in photochemotherapy of psoriasis.

The use of 8-MOP in PUVA therapy has resulted in an intense investigation of the biochemical and pharmacologic properties of psoralens. An important feature of psoralen pharmacology is the dependence of the phototoxic response on route of administration. A deeper understanding of this phenomenon is partly motivated by our need to develop compounds for use in PUVA that are therapeutically effective and yet lack the acute and chronic toxicity observed in 8-MOP therapy.

The mechanism of the therapeutic action in PUVA is still being actively investigated. The covalent photoaddition of psoralens to DNA is generally believed to be the basic event explaining the toxicologic and also the therapeutic aspects of furocoumarin photobiology. A newer approach focuses on immunologic events. We know that PUVA treatment suppresses contact hypersensitivity in experimental animals (*11*). Clinically, patients treated with PUVA show a decrease of immune reactivity and also some alteration in immune function (*12*). The role of these important events in the mechanism of PUVA still needs to be elucidated.

However, a large body of evidence still suggests that the molecular mode by which psoralens initiate a therapeutic or phototoxic response involves intercalation of the psoralen into DNA, followed by the formation of a monoadduct (upon absorption of 1 photon) or a diadduct (absorption of 2 photons). The latter lesion leads to cross-linked complementary strands of DNA. The presence and structure of this cross-link, first proposed by Cole (*13*) and Dall'Acqua et al. (*14*), has up to now been largely based on indirect evidence. Only recently have the psoralen monoadducts and diadducts been isolated and characterized by high resolution mass spectroscopy and proton nuclear magnetic resonance (*15*). This work completed a decade-long search for the structures of these important DNA photoadducts, and it emphasizes the importance of modern analytical tools in biology, toxicology, and medicine.

When the relative abilities of psoralen derivatives to intercalate and form adducts in vitro were determined, TMP was most photoreactive toward mammalian DNA,

ABBREVIATIONS: 8-MOP = 8-methoxypsoralen; 5-MOP = 5-methoxypsoralen; TMP = 4,5′,8-trimethylpsoralen; UVA = UV radiation at 320–400 nm; PUVA = psoralen plus UVA; J = joules.

[1] Presented at the Conference on Photobiologic, Toxicologic, and Pharmacologic Aspects of Psoralens held in Research Triangle Park, North Carolina, March 1–3, 1982.

[2] Animals were maintained under the guidelines set forth by the National Institutes of Health Policy on Humane Care and Use of Animals and by the Animal Welfare Act in facilities accredited by the American Association for Accreditation of Laboratory Animal Care.

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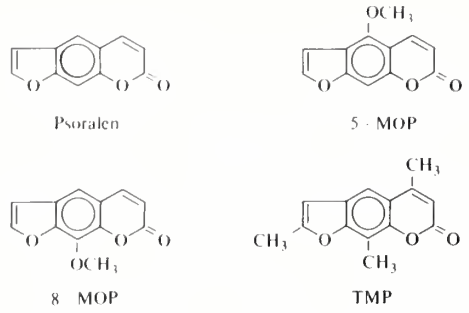

FIGURE 1.—Structures of psoralen and substituted derivatives.

followed by psoralen, 8-MOP, and then 5-MOP (*16*). These relative reactivities of psoralens closely predict several in vitro reactivities, such as inhibition of nucleic acid synthesis (*17*), photoinactivation of Ehrlich ascites tumor cells (*18*), chromosomal damage in mammalian cells (*19*), and mutagenesis in yeast (*20*).

The phototoxicity of psoralen derivatives in vivo involves an added complexity. To invoke an in vivo phototoxic reaction, the putative phototoxic agent (unmetabolized psoralen derivative) must be delivered in sufficient concentration to the target organ, the epidermis. The processes determining delivery of psoralen to epidermis following topical administration are primarily penetration into the epidermis and, possibly, cutaneous metabolism. Following oral administration of psoralens, absorption, systemic metabolism, binding to serum proteins, and distribution of psoralens to the epidermis will determine their relative oral reactivity.

It has been determined, both in humans (*21*) and animals (Kornhauser A, Wamer W, Giles A: Submitted for publication), that the structurally similar compounds 5-MOP and TMP elicit topical phototoxicity similar to 8-MOP. However, when administered orally, their phototoxicity is greatly diminished compared with that of 8-MOP (*22, 23*). Recently, this fact has been exploited by 2 European teams who have introduced 5-MOP as an alternative to 8-MOP in PUVA treatment of psoriasis (*22, 24*). Although 5-MOP and 8-MOP were comparable in their ability to clear psoriatic lesions, acute side effects (including phototoxicity) were significantly reduced in the 5-MOP regimen.

The serum levels of substituted psoralens after oral administration appear to predict the extent of phototoxicity. Following equivalent doses in humans (0.6 mg/kg), maximum serum levels of 8-MOP have been reported to range between 79 and 278 ng/ml (*25–27*), 5-MOP between 61 and 152 ng/ml (*28*), but TMP was not detectable (*29*).

These serum levels may indicate that the reduced oral phototoxicity of 5-MOP and TMP relative to 8-MOP is due to their poor absorption, and, particularly with TMP, rapid metabolism (*23*). Furthermore, the distribution of blood-borne psoralens into the epidermis may be important in determination of the relative oral phototoxicity of psoralens. Although blood levels of psoralens have been determined, these levels may not reasonably represent the level of psoralen which has diffused from superficial dermal capillaries into the epidermis. This concern may be particularly justified for short intervals following an oral dose.

We report determinations of serum and epidermal levels for 5-MOP, 8-MOP, and TMP; levels of psoralen were not determined due to the limited availability of this compound. Determinations of the levels of these derivatives in the epidermis, the primary target organ for phototoxicity, have not been reported for either humans or an animal model. Knowledge of these levels promises to provide direct insight into the aforementioned aspects of psoralen phototoxicity. For this study, we chose a guinea pig model, which has been shown by us and others (*30*), to be a reliable animal model for predicting phototoxicity in man.

MATERIALS AND METHODS

To epilate the backs of young adult albino guinea pigs of the Hartley strain, we prepared a depilatory agent by mixing 1 part beeswax, 2 parts rosin powder (with diluent), and 2 parts purified rosin lumps obtained from Fisher Scientific Company (Pittsburgh, Pa.). This mixture, at 40–48° C, was uniformly spread over the clipped area of the backs of the anesthetized guinea pigs. After approximately 5 minutes, the depilatory agent was removed. Three to 5 days after epilation, the skin returned to normal appearance, without signs of inflammation or abrasions. Each group of animals to be used for the phototoxicity studies was given either an oral dose or a topical application of the psoralen in acetone. Two hours after oral dosing or 15 minutes after topical application, the guinea pigs were immobilized on a wooden board and irradiated with 3 J UVA/cm^2, measured with an IL700 Research Radiometer from International Light Inc. (Newburyport, Mass.), UVA detector #388, and a diffuser #698, from a sun lamp filtered through glass with a cutoff of less than 315 nm. The sun lamp was fitted with a 275-W Blue Dot bulb from Sylvania Corporation (Milburn, N.J.).

We obtained the 8-MOP and TMP from Elder Pharmaceuticals (Bryan, Ohio); the psoralen was a gift from Dr. M. A. Pathak of Harvard Medical School (Boston, Mass.); and the 5-MOP was purchased from Memphis Chemical (Cairo, Egypt). The drugs were checked for identity and purity by nuclear magnetic resonance, gas chromatography, high-pressure liquid chromatography, and mass spectrometry. Oral doses, in a gelatin capsule, were given at levels listed in table 1. The 5- and 8-MOP, TMP, and psoralen were applied topically (in acetone) at 1, 10, and 100 $\mu g/cm^2$. Control guinea pigs for the oral dose received no psoralens, whereas control animals for the topical dose received acetone only and were irradiated as described. The skin was examined under uniform lighting at 24, 48, and 72 hours after irradiation. The severity of erythema was graded as 0, +, ++, +++, or ++++. To determine serum and epidermal psoralen levels, we dosed additional groups of guinea pigs orally as described above. We allowed blood samples, taken by heart puncture 2 hours after dosing, to clot at 0° C so we could obtain serum. Immediately after blood samples were drawn, the guinea pigs were killed by asphyxiation with carbon dioxide. Skin was then excised from the epilated portion (approximately 40 cm^2) of the back. To obtain epidermis,

we immersed the skin samples in 60° C distilled water for 30 seconds and then gently peeled the epidermis with forceps. To establish whether psoralens are lost in this procedure, we took duplicate skin samples from 8-MOP- or 5-MOP-dosed guinea pigs and obtained the epidermis from 1 sample after heat treatment, from the other by scraping alone. No significant loss of either 5-MOP or 8-MOP was detected from the heat-treated epidermis when compared with scraped epidermis. The weighed epidermis was then processed in distilled water in a Polytron homogenizer obtained from Brinkmann Instruments, Inc. (Westbury, N.Y.). The method of Kreuter and Higuchi (*31*) was modified for extraction of the epidermal homogenates and serum, both of which were extracted with 20% (vol/vol) methylene chloride in heptane. A 1-ml aliquot of serum diluted to 5 ml with distilled water and a 15-ml aliquot of epidermal homogenate were each spiked with an appropriate amount of internal standard, either 8-MOP or TMP, depending on the psoralen derivative to be quantitated. Concentrated hydrochloric acid was added to each "spiked sample" to a final concentration of 0.1 *N*, and samples were heated to 90° C for 1 hour. Samples were then extracted three times with equal volumes of 20% (vol/vol) methylene chloride in heptane.

Organic layers from the serum extraction were combined and evaporated to dryness under nitrogen at 60° C. Elution of epidermal extracts through a silica gel clean-up column was necessary for elimination of chromatographic impurities in the final high-pressure liquid chromatographic analysis. Epidermal extracts were loaded onto a column containing 1.5 g silica gel [5–30 μ; Supelcosil 12A, Supelco, Inc. (Bellefonte, Pa.)]. The column was eluted under nitrogen pressure with methylene chloride. Fractions containing psoralens were obtained by collection of fluorescent bands eluting from the column.

The residues obtained from each serum and epidermis extraction were reconstituted in 0.2 ml methanol and analyzed by high-pressure liquid chromatography. The chromatographic system included the Waters Associates (Milford, Mass.) Model 440 absorbance detector, Model U6K injector, Model 6000A solvent delivery system, data module, and Model 720 system controller. A Waters 3.9 mm × 30 cm C_{18}-μBondapak column was eluted with 65% (vol/vol) methanol–distilled water at 2 ml/minute.

TABLE 1.—*Phototoxicity and serum and epidermal levels of 8-MOP and 5-MOP in guinea pigs dosed orally*[a]

Compound	No. of guinea pigs	Dose level, mg/kg	Serum level, μg/ml	Epidermal level, μg/g epidermis	Erythemal grade[b]
8-MOP	4	15	1.85±0.24	1.19±0.21	++++
8-MOP	5	10	0.99±0.13	0.66±0.16	++++
8-MOP	5	5	0.30±0.04	0.22±0.04	+++
8-MOP	5	3	0.05±0.01	<0.10	0
5-MOP	5	15	0.35±0.03	0.41±0.05	+++
5-MOP	5	10	0.07±0.01	<0.10	0
TMP	2	25	<0.01	<0.10	0
TMP	2	50	<0.01	<0.10	0
Psoralen	4	15	ND	ND	0
Psoralen	4	25	ND	ND	+++

[a] Levels are expressed as average ± SE. ND = not determined.

[b] Erythemal grade was determined after 72 hr in separate experiments; dose of UVA was 3 J/cm^2.

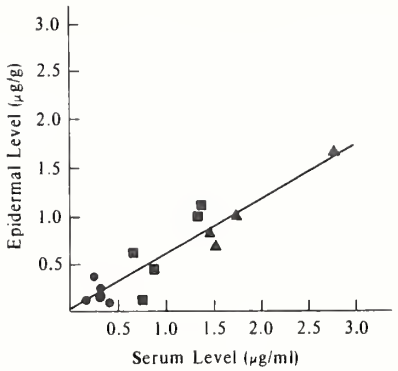

FIGURE 2.—Relationship between serum and epidermal levels of guinea pigs given 5 (●), 10 (■), and 15 (▲) mg 8-MOP/kg. Values from animals given 3 mg 8-MOP/kg are omitted because the epidermal levels were too low to quantitate. $r=0.92$; $n=14$; $P<0.001$.

RESULTS

The serum and epidermal levels, determined for each guinea pig receiving 5, 10, or 15 mg 8-MOP/kg orally, are plotted in figure 2. This correlation was not attempted for 5-MOP and TMP due to the low serum and epidermal levels encountered for these compounds. As shown, the correlation between serum and epidermal levels is high over a wide range of serum concentrations. It is clearly indicated that the 8-MOP serum level does indeed predict the level of 8-MOP in guinea pig epidermis. We made a corollary observation that, after oral administration of [^{14}C]8-MOP to guinea pigs, the disappearance of radiolabel from the epidermis parallels the decrease in blood radioactivity (unpublished results). Furthermore, we found that radiolabel in skin (as a measure of 8-MOP and metabolites) is dramatically diminished by 8 hours after the dose and closely follows the decrease in blood levels. We observed no indication of any selective accumulation of or reservoir formation for 8-MOP in guinea pig skin under our experimental conditions.

Table 1 presents data organized with respect to dose level and observed phototoxicity. Phototoxicity of 8-MOP was first observed in animals receiving 5 mg 8-MOP/kg. The appearance of phototoxicity is accompanied by an appreciable increase in serum and epidermal 8-MOP levels. We observed that the guinea pig serum levels marking the onset of phototoxicity (0.05–0.3 μg/ml) are within the range of serum levels accompanying the appearance of phototoxicity in humans receiving therapeutic doses, i.e., 0.6 mg 8-MOP/kg (*25–27*). When given either 10 or 15 mg 8-MOP/kg, all animals showed a severe phototoxic reaction with elevated serum and epidermal levels. These results in our model system indicated that 8-MOP serum levels correlate with epidermal levels and may be used to predict phototoxicity.

We found that the topical phototoxicity of 5-MOP, TMP, and psoralen in the guinea pig are similar to that of 8-MOP. However, as indicated in table 1, these psoralens are not similarly phototoxic when administered orally. The onset of phototoxicity occurs between oral doses of 10 and 15 mg/kg for 5-MOP but between 3 and 5 mg/kg for 8-MOP (table 1). We also determined that the serum and epidermal levels required for a phototoxic response are similar for both 5-MOP and 8-MOP (table 1). Thus the chief reason that 5-MOP oral phototoxicity is lower than that of 8-MOP appears to be a decreased delivery of 5-MOP to the epidermis.

In the guinea pig, we observed that TMP was not orally phototoxic in dosages up to 50 mg/kg, and serum and epidermal levels remained below detection. This is consistent with the observation that TMP is rapidly metabolized in rodents and man (*23*). The oral phototoxicity of psoralen was significantly less than 8-MOP, and the onset ranged between 15 and 25 mg/kg. Determinations of serum and epidermal levels for psoralen are in progress.

DISCUSSION

The fact that psoralens, as used in photochemotherapy, react covalently with DNA poses a potential risk of mutagenicity and oncogenicity. The relative mutagenicity and carcinogenicity of various psoralen derivatives have only been partially studied. In an in vitro study, 8-MOP and 5-MOP exhibited essentially the same activity in inducing chromosome damage in human cells (*19*). Furthermore, topical 5-MOP in combination with UVA induces carcinogenesis in mice comparable to that induced by 8-MOP (*32*). These 2 studies suggest that 5- and 8-MOP have similar oncogenic potential. The corresponding data for TMP and psoralen are far less complete. Such a toxicologic study would be extremely valuable, particularly because the photoreactivity of TMP toward DNA greatly exceeds that of other naturally occurring psoralens (*16*).

Factors in addition to DNA photoreactivity will moderate the in vivo toxic or therapeutic effects of psoralens. For example, our results indicate that, when equivalent oral doses are given, 5-MOP is present at lower epidermal levels than 8-MOP due to a difference in their metabolism or absorption, or both. This means that by orally administering 5-MOP, it should be possible for one to maintain epidermal drug concentrations at lower levels than in an 8-MOP regimen. In extrapolating these findings to clinical applications, one could find that a 5-MOP therapeutic regimen may minimize damage to epidermal DNA, thereby reducing the risk of carcinogenesis, which is strongly suspected in 8-MOP photochemotherapy (*33*), and yet be effective in inhibiting rapidly proliferating psoriatic cells. The European experience with 5-MOP therapy shows that phototoxicity is not required for a remedial effect. For this reason and because of the reported reduced acute side effects in a 5-MOP regimen (*22*), we recommend that 5-MOP be tested further with other psoralen derivatives as an alternative to 8-MOP in PUVA.

The increased use of psoralens in PUVA emphasizes our urgent need to know and understand the pharmacologic behavior and mode of action of these compounds. Experimental studies of the type reported can provide important information about the oral phototoxicity of psoralens in addition to research on their metabolism and excretion. We believe that monitoring serum and epidermal levels will be of great value in providing a better understanding of oral phototoxicity due to foods and drugs and also to develop new compounds for photochemotherapy.

REFERENCES

(*1*) Berenbaum M, Feeny P: Toxicity of angular furanocoumarins to swallow tail butterflies: Escalation in a coevolutionary arms race? Science 212:927–929, 1981

(*2*) Johnson C, Brannon DR: Xanthotoxin: A phytoalexin of *Pastinaca sativa* root. Phytochemistry 12:2961–2962, 1973

(*3*) Scheel LD, Perone VB, Larkin RL, et al: The isolation and characterization of two phototoxic furanocoumarins from diseased celery. Biochemistry 285:1127–1131, 1980

(*4*) Birmingham DJ, Key MM, Tubich GE, et al: Phototoxic bullae among celery harvesters. Arch Dermatol 83:73–87, 1961

(*5*) Marzulli F, Maibach H: Perfume phototoxicity. J Soc Cosmet Chem 21:695–715, 1970

(*6*) Ashwood-Smith MJ, Poulton GA, Barker M, et al: 5-Methoxypsoralen, an ingredient in several suntan preparations has lethal, mutagenic and clastogenic properties. Nature 285:407–409, 1980

(*7*) Ivie GW, Holt DL, Ivey MC: Natural toxicants in human foods: Psoralens in raw and cooked parsnip root. Science 213:909–910, 1981

(*8*) Fitzpatrick TB, Pathak MA: Historical aspects of methoxsalen and other furocoumarins. J Invest Dermatol 32:229–231, 1959

(*9*) Gilchrest BA, Parrish JA, Tanenbaum L, et al: Oral methoxsalen photochemotherapy of mycosis fungoides. Cancer 38:683–689, 1976

(*10*) Parrish JA, Fitzpatrick TB, Tanenbaum L, et al: Photochemotherapy of psoriasis with oral methoxsalen and longwave ultraviolet light. N Engl J Med 291:1207–1211, 1974

(*11*) Morison WL, Woehler ME, Parrish JA: PUVA and systemic immunosuppression in guinea pigs. J Invest Dermatol 72:273, 1979

(*12*) Morison WL, Parrish JA, Block KJ, et al: Transient impairment of peripheral blood lymphocyte function during PUVA therapy. Br J Dermatol 101:391–397, 1979

(*13*) Cole RS: Light-induced cross-linking of DNA in the presence of a furocoumarin (psoralen). Biochim Biophys Acta 217:30–39, 1970

(*14*) Dall'Acqua F, Marciani S, Ciavatta L, et al: Formation of inter-strand cross-linkings in the photoreactions between furocoumarins and DNA. Z Naturforsch [C] 266:561–569, 1971

(*15*) Isaacs S, Hearst JE: In vitro characterization of the reaction of four psoralens and psoralen derivatives with DNA. Natl Cancer Inst Monogr 66:21–30, 1984

(*16*) Musajo L, Rodighiero G, Caporale G, et al: Photoreactions between skin-photosensitizing furocoumarins and nucleic acids. *In* Sunlight and Man: Normal and Abnormal Photobiologic Responses (Fitzpatrick TB, Pathak MA, Harber LC, et al, eds). Tokyo: Univ Tokyo Press, 1974, pp 369–388

(*17*) Bordin F, Baccichetti F, Musajo L: Inhibition of nucleic acids synthesis in Ehrlich ascites tumor cells by irradiation in vitro in the presence of skin-photosensitizing furocoumarins. Experientia 28:148, 1972

(*18*) MUSAJO L, VISENTINI P, BACCICHETTI F, et al: Photoinactivation of Ehrlich ascites tumor cells in vitro obtained with skin-photosensitizing furocoumarins. Experientia 15: 335–336, 1967

(*19*) NATARAJAN AT, VERDEGAAL-IMMERZEEL EA, ASHWOOD-SMITH MJ, et al: Chromosomal damage induced by furocoumarins and UVA in hamster and human cells including cells from patients with ataxia telangiectasia and xeroderma pigmentosum. Mutat Res 84:113–124, 1981

(*20*) AVERBECK D, AVERBECK S, DALL'ACQUA F: Mutagenic activity of three monofunctional and three bifunctional furocoumarins in yeast (*Saccharomyces cerevisiae*). Farmaco [Sci] 36:492–505, 1971

(*21*) PATHAK MA, FITZPATRICK TB: Relationship of molecular configuration to the activity of furocoumarins which increase the cutaneous responses following long wave ultraviolet radiation. J Invest Dermatol 32:255–262, 1959

(*22*) HÖNIGSMANN H, JASCHKE E, GSCHNAIT F, et al: 5-Methoxypsoralen (bergapten) in photochemotherapy of psoriasis. Br J Dermatol 101:369–378, 1979

(*23*) MANDULA BB, PATHAK MA: Photochemotherapy: Identification of a metabolite of 4,5′,8-trimethylpsoralen. Science 193:1131–1134, 1976

(*24*) GRUPPER C, BERRETT B: 5-MOP in PUVA and Re-PUVA—a monocentric study: 250 Patients with a follow-up of three years. Presented at the Third International Symposium on Psoriasis, Stanford, Calif., 1981

(*25*) THUNE P, VOLDEN G: Photochemotherapy of psoriasis with relevance to 8-methoxypsoralen plasma level and low intensity radiation. Acta Derm Venereol (Stockh) 57: 351–355, 1977

(*26*) STEINER I, PREY T, GSCHNAIT F, et al: Serum levels of 8-methoxypsoralen 2 hours after oral administration. Acta Derm Venereol (Stockh) 58:185–188, 1978

(*27*) LJUNGGREN B, CARTER DM, ALBERT J, et al: Plasma levels of 8-methoxypsoralen determined by high-pressure liquid chromatography in psoriatic patients ingesting drug from two manufacturers. J Invest Dermatol 74:59–62, 1980

(*28*) STOLK L, WESTERHOF W, CORMANE RH, et al: Serum and urine concentrations of 5-methoxypsoralen after oral administration. Br J Dermatol 105:415–420, 1981

(*29*) STOLK L, SIDDIQUI AH, CORMANE RH: Serum levels of trimethylpsoralen after oral administration. Br J Dermatol 104:443–445, 1981

(*30*) HARBER LC: Use of guinea pigs in photobiologic studies. *In* The Biologic Effects of Ultraviolet Radiation (Urbach F, ed). New York: Pergamon Press, 1969, pp 291–293

(*31*) KREUTER J, HIGUCHI T: Improved delivery of methoxsalen. J Pharm Sci 68:451–453, 1979

(*32*) ZAJDELA F, BISAGNI E: 5-Methoxypsoralen, and melanogenic additive in sun-tan preparations, is tumorigenic in mice exposed to 365 nm UV radiation. Carcinogenesis 2:121–127, 1981

(*33*) STERN RS, THIBODEAU LA, KLEINERMAN RA, et al: Risk of cutaneous carcinoma in patients treated with oral methoxsalen photochemotherapy for psoriasis. N Engl J Med 300:809–813, 1979

DISCUSSION

R. Brickl: Dr. Kornhauser, you said that there is not much difference observed with topical administration of TMP, 5-MOP, and 8-MOP.

A. Kornhauser: Yes, I did.

Brickl: Well, I must say that in humans, we found a considerable difference between TMP, which is not only much stronger but also starts acting earlier after the treatment, and the other 2 compounds. Thus it might be that in human skin, the limiting factor (and we have some evidence for it) is absorption, which means penetration into the skin layers where the effect may occur.

Kornhauser: First of all, we used epilated guinea pigs. Although we wait a few days after epilation before using the animals, the stratum corneum is still largely missing due to the epilation procedure. The skin penetration demonstrated by a human being, who is not epilated and therefore has an intact stratum corneum, may be expected to be different.

Brickl: You had problems of needing to give higher doses of 5-MOP orally. It is much more difficult to obtain plasma levels, as you found out, if one gives crystalline material with 5-MOP than with 8-MOP.

Kornhauser: Absolutely. Particularly with TMP we could not obtain plasma levels at all after dosing orally with crystalline compound at dose levels up to 50 mg/kg. It is possible that at this dose level we might have found detectable levels if TMP had been given in solution.

Brickl: How did you do the skin assay?

Kornhauser: We obtained the epidermis by heat treatment of the whole skin. The epidermis was homogenized and the homogenate was extracted. The extract went through a purification phase and then was analyzed by high-pressure liquid chromatography.

Chemical Characterization of Psoralens Used in the National Toxicology Program Research Projects[1, 2]

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ABSTRACT—Chemical analyses of 4 psoralen derivatives were performed with the use of various techniques, including thin-layer chromatography, gas chromatography, infrared and nuclear magnetic resonance spectroscopy. 8-Methoxypsoralen, 5-methoxypsoralen, 3-carbethoxypsoralen, and 5-methylisopsoralen were determined to be 99, 93, 97, and 99% pure, respectively. A 7% contaminant in the 5-methoxypsoralen sample was identified as a dimethoxypsoralen isomer. These psoralens are being used in research projects sponsored by the National Toxicology Program. — Natl Cancer Inst Monogr 66: 103–113, 1984.

The National Toxicology Program has sponsored several research projects for the determination of the toxicologic properties of psoralen derivatives and UV light (UVA). Psoralens in combination with UVA are used in the treatment of various skin diseases including vitiligo and psoriasis (*1–3*). The exact mechanism of action for the treatment of these diseases is not known, but the psoralen, in combination with UVA, is thought to inhibit DNA synthesis (*4–6*). The ability of the psoralen and UVA to interact with DNA may also cause undesirable side effects. A major part of this research has been on the chemical characterization of several psoralens and analyses of their purity. We performed these analyses to ensure high purity of the chemicals and to enable other scientists to compare their test materials with those used in the National Toxicology Program. Of the psoralens, 8-MOP has been the most widely used in clinical studies in the United States (*1, 2*), and this is the only one approved for use by the Food and Drug Administration. Therefore, in these studies, 8-MOP was considered to be the standard psoralen. Other psoralens studied by the Program include 5-MOP, 3-CP, and 5-MIP.

MATERIALS AND METHODS

Chemicals.—The 8-MOP (Lot #21900) was procured from Elder Pharmaceuticals (Bryan, Ohio); the 5-MOP (Lot #T032681) was supplied by Dr. P. Donald Forbes, Temple University (Philadelphia, Pa.) and prepared by the Memphis Chemical Company (Zeitoun, Egypt); 3-CP (Lot #T032681) was prepared by Prof. R. Latarjet, Fondation Curie, Institut du Radium (Paris, France); and 5-MIP (Lot #H110381) was synthesized by HRI Associates [(Emeryville, Calif.); *see* fig. 1 for structures of the chemicals].

Chemicals analyses.—Purity analysis for all chemicals was conducted at Midwest Research Institute (Kansas City, Mo.). Purity was determined by various methods, including elemental and spectral analyses and chromatography. The specific gas chromatographic conditions are outlined in tables 1 and 2. Proton nuclear magnetic resonance spectra were obtained on a 60-megahertz EM360-A spectrometer from Varian Associates (Palo Alto, Calif.). The IR spectrum analysis of potassium bromide pellets of the material was done with a No. 283 spectrometer from Perkin-Elmer Corporation (Norwalk, Conn.). A Cary 219 spectrophotometer, also from Varian Associates, was used for the UV analysis, with ethanol as the solvent.

RESULTS

8-Methoxypsoralen

Elemental analyses for carbon and hydrogen agreed with the theoretical values; Karl Fischer titration for water indicated less than 0.1%. Free acid (as the hydrolyzed lactone) titration indicated a concentration of less than 0.1%. Compendial UV spectrometric assay at 300 nm (USP XIX, 317–318) revealed a relative purity of $98.8 \pm 3.2\%$ (relative standard deviation) versus a USP reference standard. Thin-layer chromatography indicated a major spot and a trace origin spot with solvent system 1, whereas solvent system 2 detected a major spot only (table 1). A USP reference standard of 8-MOP spotted concomitantly with test samples showed a major spot identical to that for the sample in solvent system 1; a slight trace above the major spot was observed for the standard in solvent system 2. Gas chromatographic analysis by the 2 systems listed in table 2 indicated a major peak and 1 impurity peak which had an area of 0.14 and 0.12% of the major peak area in systems 1 and 2, respectively. The IR (fig. 2), UV/visible, and nuclear magnetic resonance spectra (fig. 3) were consistent with the structure and literature references (*7, 9*). The combined data indicated that the test material had a purity of greater than 99% and was consistent with USP purity requirements.

5-Methoxypsoralen

Because the sample size was limited, elemental analysis and free acid titration were not performed. Thin-layer chromatography, with the 2 solvent systems listed in table 1, resolved a major spot only. With the use of a 1% SP-1000

ABBREVIATIONS: UVA = ultraviolet radiation at 320–400 nm; 8-MOP = 8-methoxypsoralen; 5-MOP = 5-methoxypsoralen; 3-CP = 3-carbethoxypsoralen; 5-MIP = 5-methylisopsoralen; MHz = megahertz; ppm = parts per million.

[1] Presented at the Conference on Photobiologic, Toxicologic, and Pharmacologic Aspects of Psoralens held in Research Triangle Park, North Carolina, March 1–3, 1982.

[2] This is National Toxicology Program Document No. 82-096.

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TABLE 1.—*Thin-layer chromatography conditions used for psoralen analyses*[a]

Chemical	Solvent system	R_F
8-MOP	1) Diethyl ether (anhydrous)	Major: 0.403; trace: origin; Ref: 0.510
	2) Hexane:ethyl acetate:methanol (65:26:9)	Major: 0.465; Ref: 0.592
5-MOP	1) Ethyl acetate:hexane:acetic acid (85:13:2)	Major: 0.54; Ref: 0.60
	2) Toluene:acetone:acetic acid (80:18:2)	Major: 0.42; Ref: 0.46
3-CP	1) Ethyl acetate:hexane:acetic acid (85:13:2)	Trace: 0.64; Major: 0.57[b] Trace: 0.36, Ref: 0.62[b]
	2) Toluene:acetone:acetic acid (80:18:2)	Major: 0.38, Trace: 0.25[b] Ref: 0.47
5-MIP	1) Toluene:acetonitrile (90:10)	Major: 0.37, Trace: 0.29[b] Trace: Origin, Ref: 0.36[b]
	2) Chloroform:methanol (97:3)	Major: 0.62, Ref: 0.60

[a] Silica gel 60, F-254 plates were used; references standard was coumarin; and visualization was with UV at 254 or 366 nm.
[b] Trace amounts were detected when 366 nm were used for visualization.

column, gas chromatography resolved a major peak and 1 impurity that eluted after the major component, with a peak area of 7.3% relative to the major peak. The second gas chromatographic system (3% Dexsil 400) resolved a major peak and 1 impurity with a relative peak area of 6.6% that also eluted after the major component. This impurity was identified by gas chromatography/mass spectrometry with a model No. 4000 mass spectrometer interfaced to a Finnigan 9610 gas chromatograph, both obtained from Finnigan MAT (San Jose, Calif.). The 7% impurity was identified on the basis of its mass spectra as an isomer of dimethoxypsoralen. The IR (fig. 4) and nuclear magnetic resonance spectra (fig. 5) were consistent with the structure and literature references (*10, 11*). The cumulative data indicated the test chemical was approximately 93% pure and contained a single impurity of approximately 7% that was identified by gas chromatography/mass spectrometry as an isomer of dimethoxypsoralen.

3-Carbethoxypsoralen

Again, because only a small amount of compound was available, elemental analysis and free acid titration were not performed. With the use of solvent system 1 (table 1), thin-layer chromatography resolved a major spot and 2 trace impurities, whereas solvent system 2 (table 1) resolved a major peak and a single trace impurity. No impurities were observed by gas chromatography when the 2 columns listed in table 2 were used. The IR spectrum (fig. 6) was consistent with the structure of 3-CP. The chemical shifts observed by proton nuclear magnetic resonance (fig. 7) recorded in deuterochloroform were as follows:

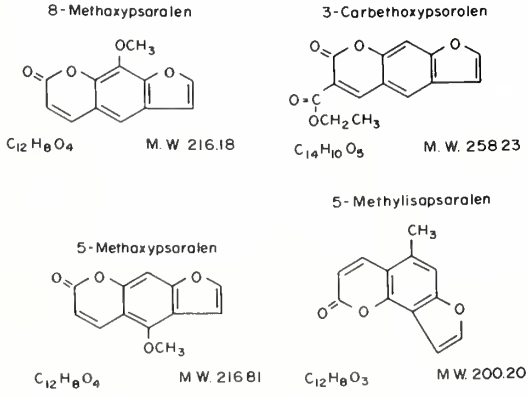


FIGURE 1.—Chemical structures of the 4 psoralen derivatives studied.

Proton	Chemical shift, ppm	Integration	
		Observed	Theoretical
H-a	1.44	2.98	3
H-b	4.42	1.93	2
H-c	6.89	1.00	1
H-d	7.44	1.02	1
H-e	7.75	2.05	2
H-f	7.85		
H-g	8.65	1.02	1
H-h	1.97 impurity	0.10	—

TABLE 2.—*Gas chromatographic conditions used for psoralen analyses*

Instrument	Varian 3700
Detector	Flame ionization
Inlet temperature	200° C
Detector temperature	250° C
Oven temperature program	50° C for 5 min, then 50 to 250° C at 10° C/min
Carrier gas	Nitrogen
Column dimensions	1.8 m × 4 mm inner diameter glass
Injection volume	5 ml of 0.5% (weight/vol) or 1% (weight/vol) of psoralen derivative in chloroform

Chemical	Column I	Column II
8-MOP	3% SP-2100 on 100/120 Supelcoport	1% SP-1000 on 100/120 Supelcoport
5-MOP	1% SP-1000 on 100/120 Supelcoport	3% Dexsil 400 on 100/120 Supelcoport
3-CP	3% SP-2100 on 100/120 Supelcoport	3% Dexsil 400 on 100/120 Supelcoport
5-MIP	3% SP-2100 on 100/120 Supelcoport	3% SP-2401 on 100/120 Supelcoport

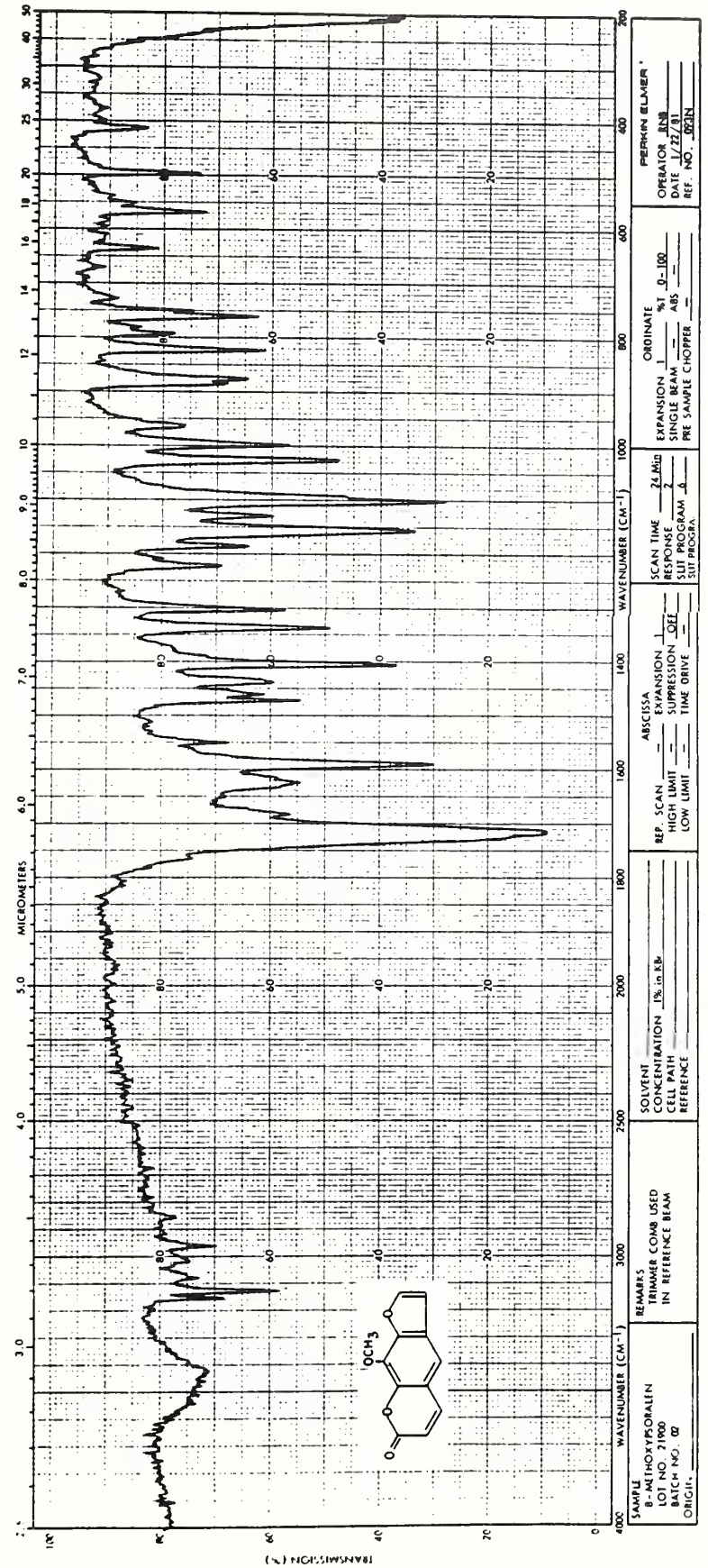
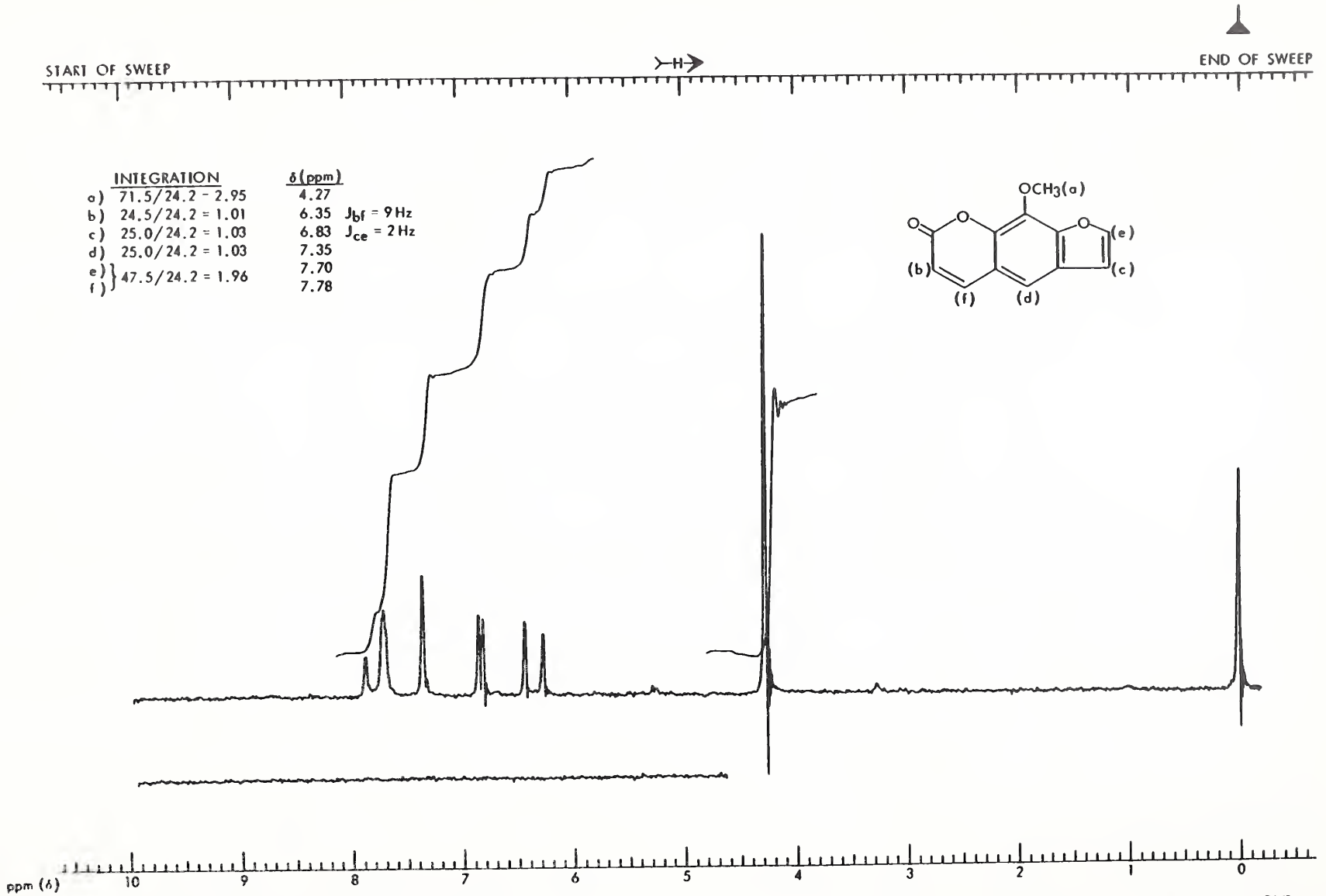


FIGURE 2.—IR spectrum of 8-MOP.



EM-360 60 MHz NMR SPECTROMETER

FIGURE 3.—Nuclear magnetic resonance spectrum of 8-MOP.

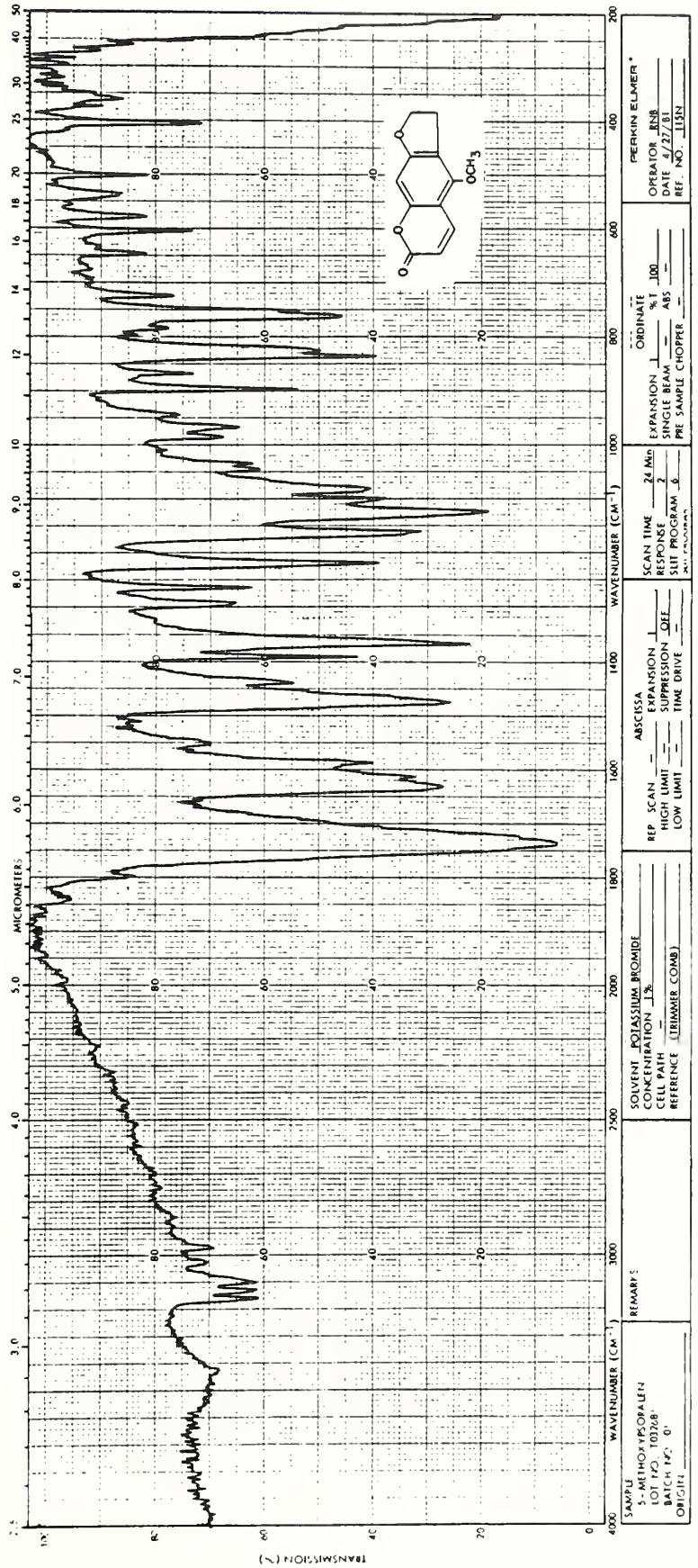


FIGURE 4.—IR spectrum of 5-MOP.

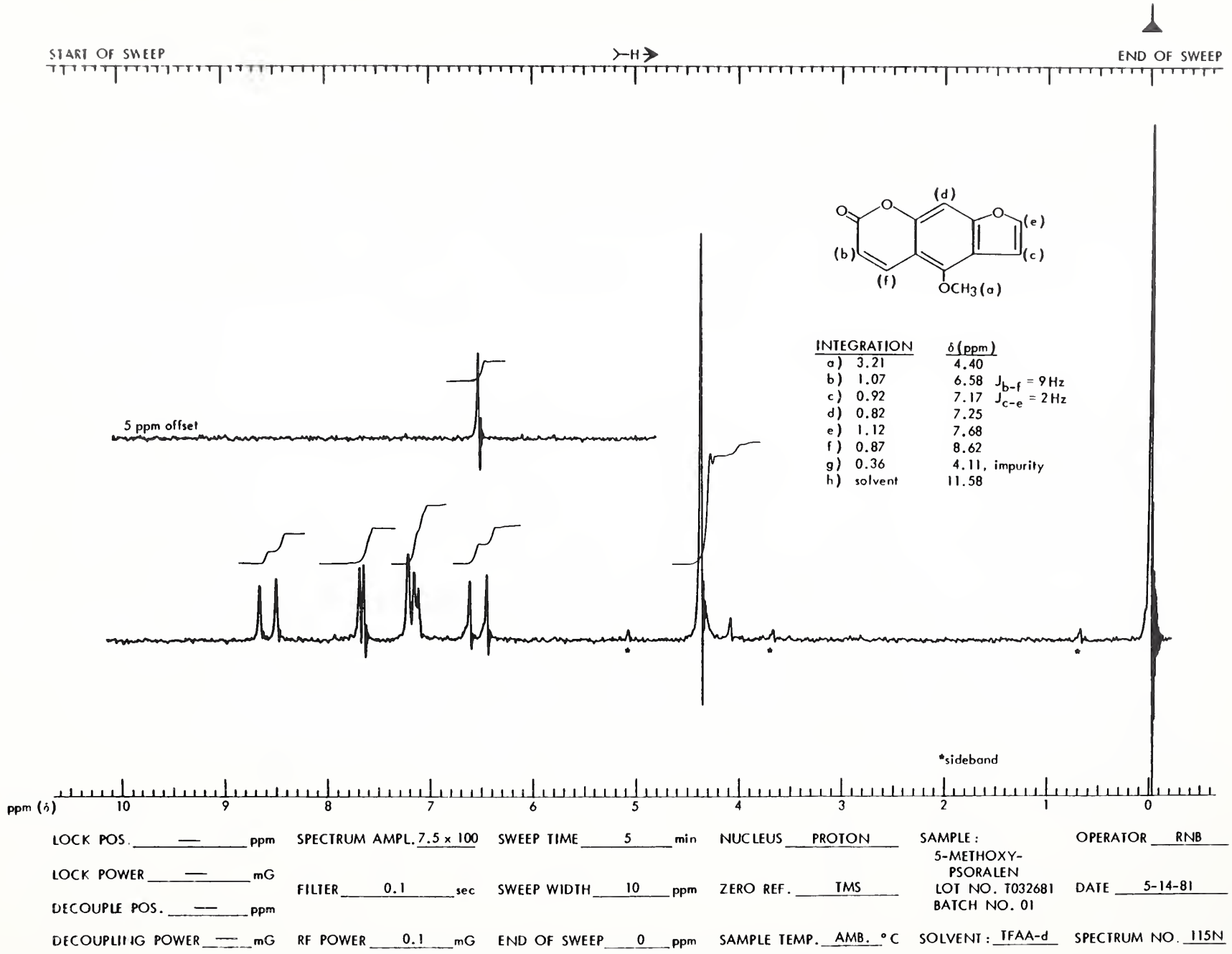


FIGURE 5.—Nuclear magnetic resonance spectrum of 5-MOP.

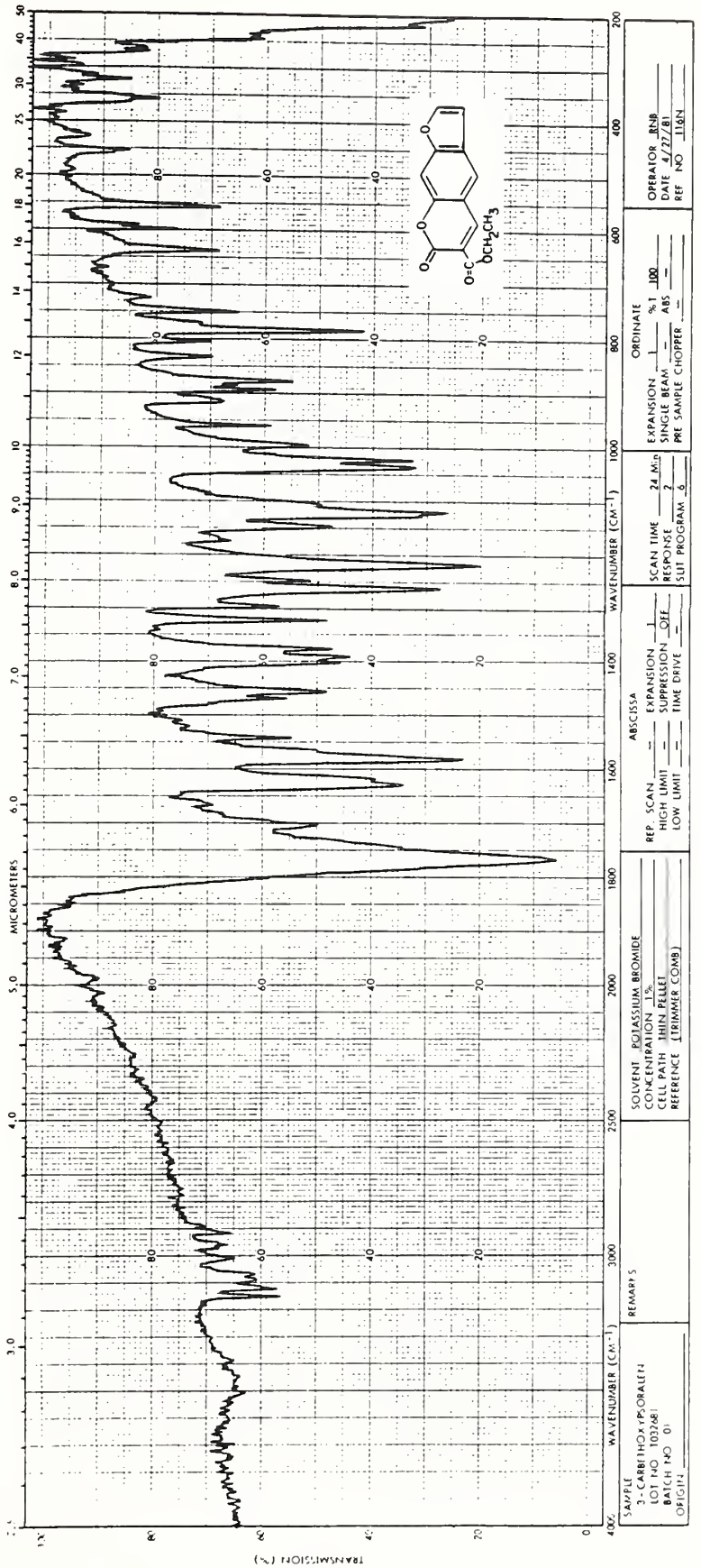
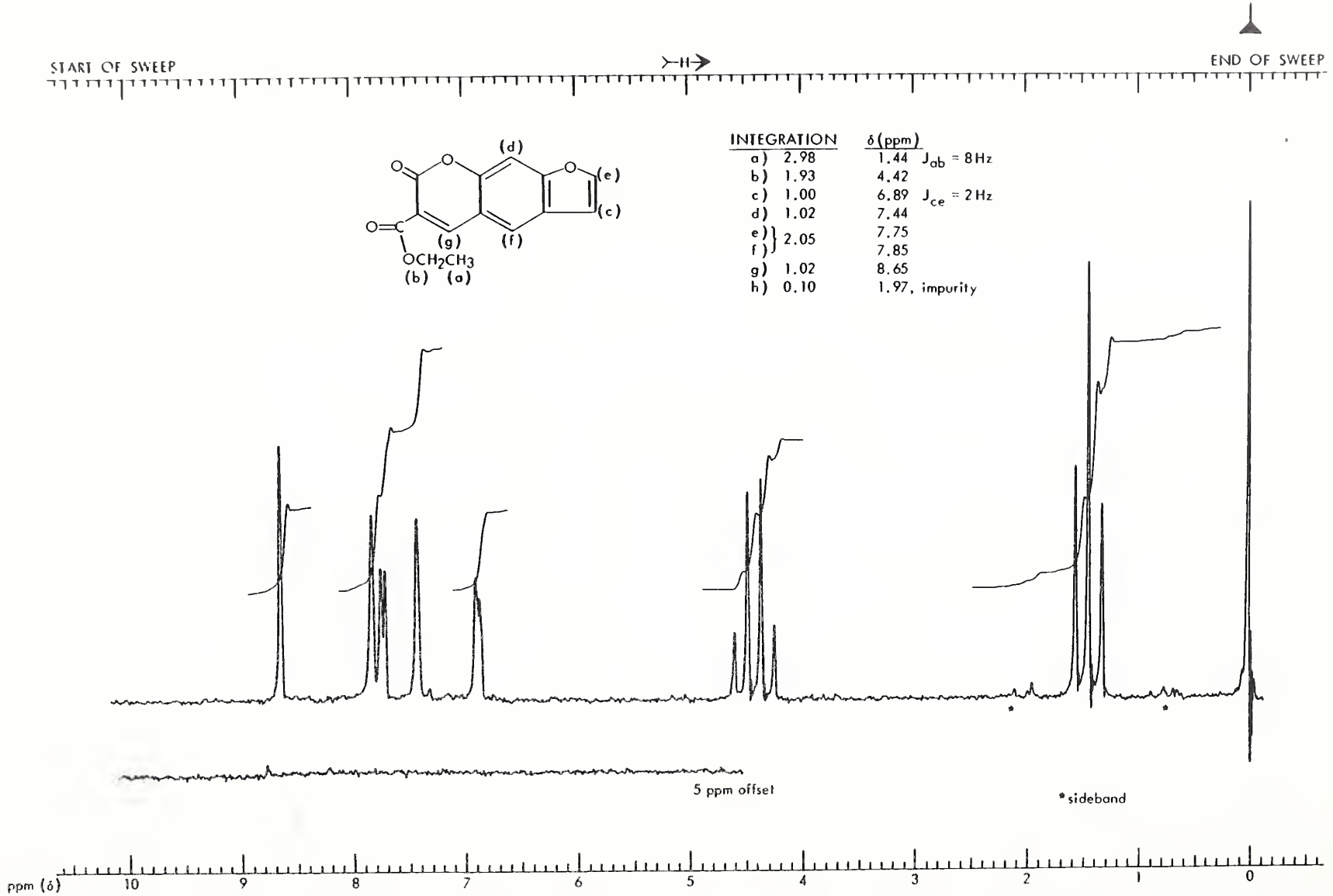


FIGURE 6.—IR spectrum of 3-CP.



EM-360 60 MHz NMR SPECTROMETER

FIGURE 7.—Nuclear magnetic resonance spectra of 3-CP.

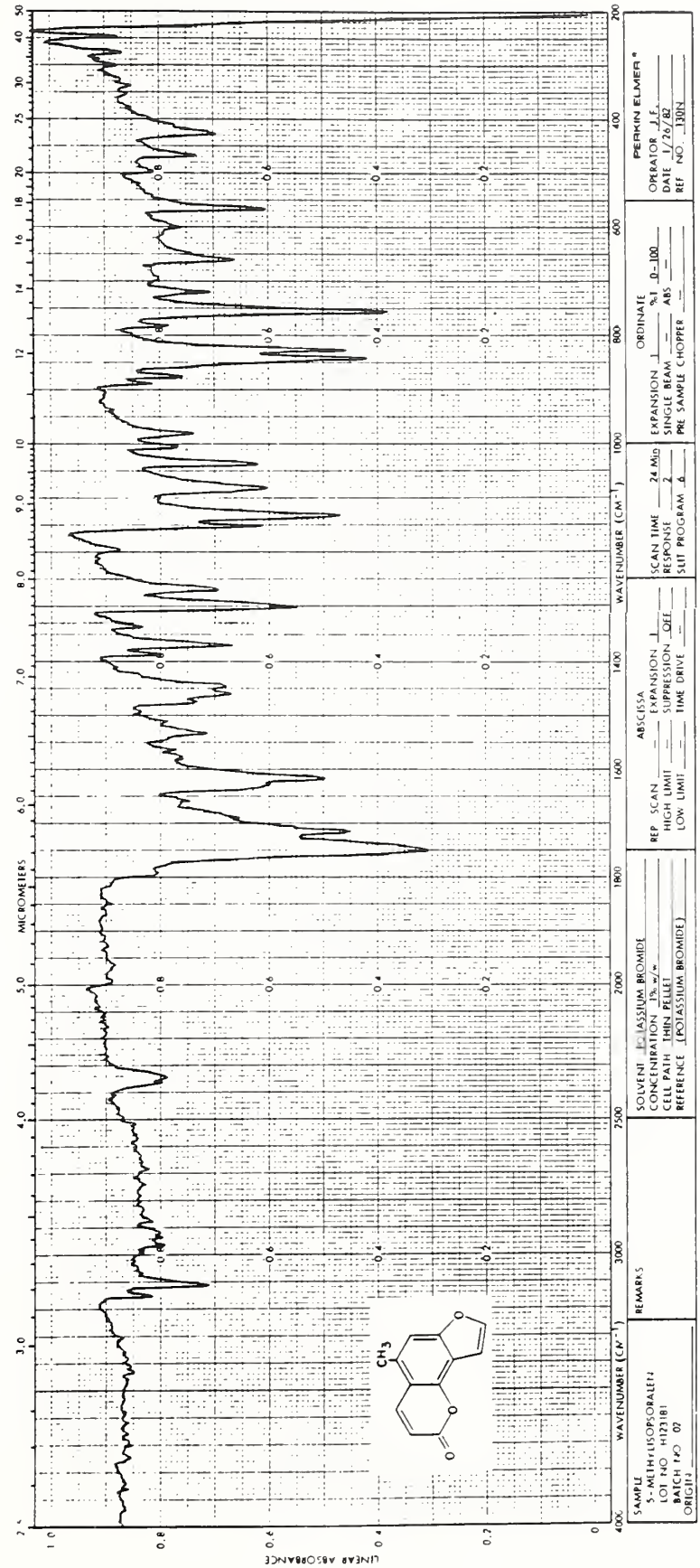


FIGURE 8.—IR spectrum of 5-MIP.

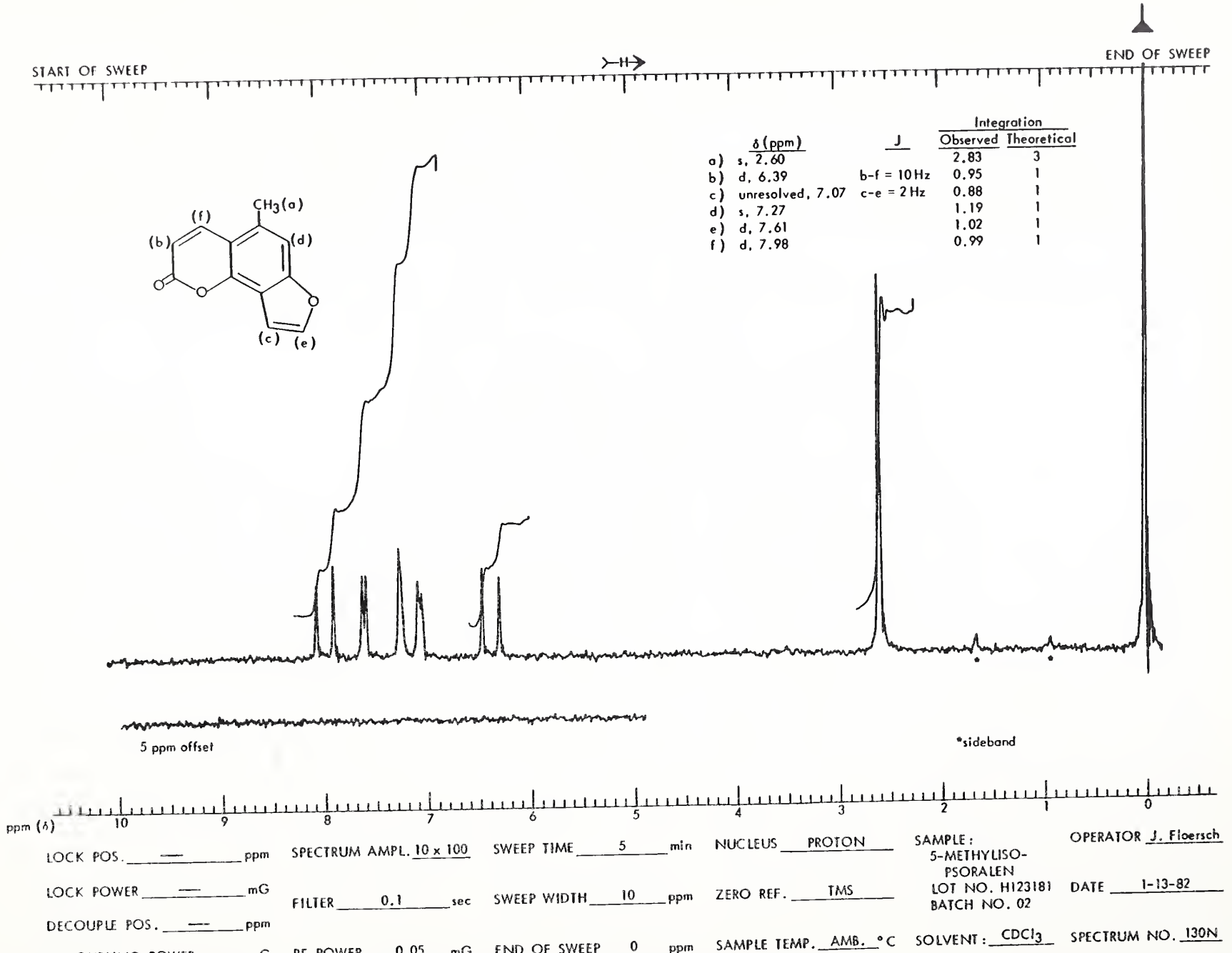


FIGURE 9.—Nuclear magnetic resonance spectra of 5-MIP.

On the basis of expected levels of detection by thin-layer chromatography and the absence of impurities by gas chromatography, the purity of the test material is estimated to be greater than 97%.

5-Methylisopsoralen

Elemental analysis and free acid titration of 5-MIP were not performed because the amount of test material was limited. When solvent system 1 (table 1) was used, thin-layer chromatography resolved a major spot and 2 trace impurities (1 at the origin). Solvent system 2 (table 1) resolved a major spot only. The 2 gas chromatographic systems (table 2) each resolved a major peak only. The IR spectrum (fig. 8) was consistent with the structure of 5-MIP. The chemical shifts observed by proton nuclear magnetic resonance (fig. 9) recorded in deuterochloroform were as follows:

Proton	Chemical shift, ppm	Integration	
		Observed	Theoretical
H-a	2.60	2.83	3
H-b	6.39	0.95	1
H-c	7.07	0.88	1
H-d	7.27	1.19	1
H-e	7.61	1.02	1
H-f	7.98	0.99	1

These data indicate that the test material has a purity greater than 99%.

CONCLUSIONS

The results of the chemical analyses of 8-MOP, 5-MOP, 3-CP, and 5-MIP show that these chemicals were all high purity materials and met the specifications of the National Toxicology Program for use in toxicity testing. It was important for us to know the purity of each psoralen used so that the relative potency for each could be compared on a molarity basis. These psoralens are being used in studies of metabolism and genetic testing and those of chronic toxicity sponsored by the National Toxicology Program.

The chemical analyses were accomplished with several standard analytical techniques including chromatography and spectrometry. The IR and nuclear magnetic spectra given for each of the psoralens will aid scientists in other laboratories to identify other psoralens. The most useful techniques for the determination of the purity of the chemicals were gas and thin-layer chromatography, which are widely used by investigators working in most laboratories. As new psoralens are developed, their chemical properties can be compared with those of the 4 psoralens used in these studies.

REFERENCES

(1) PARRISH JA, FITZPATRICK TB, TANENBAUM L, et al: Photochemotherapy of psoriasis with oral methoxsalen and longwave ultraviolet light. N Engl J Med 291:1207–1211, 1974

(2) STERN RS, THIBODEAU LA, KLEINERMAN RA, et al: Risk of cutaneous carcinoma in patients treated with oral methoxsalen photochemotherapy for psoriasis. N Engl J Med 300:809–813, 1979

(3) PARRISH JA, FITZPATRICK TB, SHEA C, et al: Photochemotherapy of vitiligo. Use of orally administered psoralens and a high-intensity long-wave ultraviolet light system. Arch Dermatol 112:1531–1534, 1976

(4) PATHAK MA, KRÄMER DM, FITZPATRICK TB: Photobiology and photochemistry of furocoumarins (psoralens). *In* Sunlight and Man: Normal and Abnormal Photobiologic Responses (Fitzpatrick TB, Pathak M, Harber LC, eds). Tokyo: Univ Tokyo Press, 1974, pp 335–368

(5) GOYAL GC, GROSSWEINER LI: The effect of DNA binding on initial 8-methoxypsoralen photochemistry. Photochem Photobiol 29:847–850, 1979

(6) DALL'ACQUA F, VEDALDI D, BORDIN F: New studies on the interaction between 8-methoxypsoralen and DNA in vitro. J Invest Dermatol 73:191–197, 1979

(7) SIMONS WW, ZANGER M: Sadtler Guide to NMR Spectra. Philadelphia: Sadtler Res Laboratories, 1972 (IR No. R413)

(8) WEAST RJ, ASTLE MJ, BEYER WH (eds): Handbook of Chemistry and Physics (56th ed). Boca Raton, Fla.: CRC Press, 1976, p C-539

(9) WINDHOLZ M, BUDAVARI S, STROUMTSOS L (eds): The Merck Index (9th ed). Rahway, N.J.: Merck & Co., Inc., 1976, p 783

(10) ABU-MUSTAFA EA, FAYEZ MB: Natural coumarins. VI. Nuclear magnetic resonance spectra of some coumarin and coumarilic acid derivatives. Can J Chem 45:325–327, 1967

(11) LEE KH, SIONE TO: Coumarins. X. Spectral studies on some linear furanocoumarins. J Pharm Sci 58:681–683, 1969

SESSION IV

Phototoxic, Mutagenic, and DNA Repair Aspects of Psoralen and Longwave Radiation Therapy

Cutaneous Phototoxicity Due to Psoralens[1]

Richard W. Gange *and* John A. Parrish[2]

ABSTRACT—Psoralen phototoxicity has several features which distinguish it from other cutaneous responses to UV radiation, with or without an exogenous photosensitizing agent. Erythema resulting from psoralen phototoxicity shows a longer latent period between irradiation and onset, during which no visible cutaneous changes are present. The dose-response curve is steeper, with blistering reactions occurring in some subjects after as little as three to four times the minimum phototoxic dose of UV radiation at 320–400 nm (UVA). The acute phase of psoralen phototoxicity is followed by a more marked increase in epidermal pigmentation than is seen after most other phototoxic reactions or following UV irradiation alone. The pathways leading to the development of cutaneous phototoxicity have not been identified. The importance of the cross-linking of DNA as the initiating event is suggested but not proved by comparative data on different psoralen compounds with different cross-linking abilities and by wavelength-dependent selective photochemistry. The subsequent pathways leading to erythema and the mediators which are liberated have not been identified. In contrast to erythema induced by UVA and UV at 290–320 and at 220–290 nm (UVB and UVC, respectively), no evidence for the involvement of prostaglandins has been demonstrable. Histopathologic studies show changes in the epidermis and dermis, with damage to keratinocytes and an inflammatory infiltrate in the dermis, both of which occur later and are of longer duration than the damage induced by UVA, UVB, or UVC alone. Despite the widespread application of psoralen phototoxicity in humans in the form of PUVA treatment, much work remains to be done before we can elucidate the important mechanisms and pathways leading to the inflammatory and therapeutic responses which are induced in the skin. Improvement of our knowledge in this area is central to the evolution of safer and more effective forms of photochemotherapy. — Natl Cancer Inst Monogr 66: 117-126, 1984.

PSORALEN PHOTOTOXICITY

The term "phototoxicity" will be used here to denote damage to biologic materials by the interaction of photons with an exogenous chemical (photosensitizer). Different usage of the term in different contexts makes it necessary to define its scope. The term "phototoxicity" has also been applied to damage induced by photons without a photosensitizer, e.g., the sunburn reaction or the response to artificial UVB irradiation. Although this usage is etymologically justifiable, we will use the term here only when an identified exogenous phototoxic compound is present.

In cutaneous medicine, abnormal skin sensitivity to light, or photosensitivity, when induced by an exogenous agent, is usually subdivided into *photoallergy* and *phototoxicity*. Photoallergy implies participation of the immune system in a cutaneous response to the exogenous agent and light. The development of specifically sensitized immunocompetent cells results in a cutaneous hypersensitivity response and persons would not be expected to be photosensitive upon first exposure to the agent. Although some potent photoallergens have the ability to sensitize allergically the majority of exposed individuals, in most instances only a small fraction of those exposed develop a cutaneous immunologic response. In contrast, phototoxicity does not involve the immune system, and, under appropriate exposure conditions, all individuals will respond with a phototoxic reaction to each exposure including the first. Psoralens are phototoxic rather than photoallergic compounds, although rare instances of photoallergy have been described. Although the distinction between photoallergy and phototoxicity is clear in principle, in practice many agents have the capacity to photosensitize by both mechanisms and clinical distinction may be problematic. Few compounds have been studied in sufficient detail for investigators to define the mechanisms adequately.

In mammalian skin, the most striking features of acute phototoxicity due to psoralens are delayed erythema and pigmentation. Under appropriate conditions of exposure and dosage, edema and blistering may also be present. The cutaneous response that has excited the greatest interest in recent years is the therapeutic response of various skin diseases to repeated exposure of diseased skin to psoralen and UV radiation of appropriate wavelengths or PUVA. Despite the extensive experience which has now been accumulated in the use of this therapy, particularly in psoriasis, the relationship between the therapeutic response and phototoxicity as it occurs in normal skin is unknown. Similarly, despite the extensive knowledge regarding photoreactions of psoralens with a wide variety of biologic substrates, the photochemical events most important in initiating the sequence of reactions leading to visible cutaneous phototoxicity are also unknown. However, comparative data on different psoralen molecules and their

ABBREVIATIONS: UVB = UV radiation at 290–320 nm; PUVA = psoralen plus UVA at 320–400 nm; 8-MOP = 8-methoxypsoralen; MPD = minimum phototoxic dose(s); TMP = 4,5′,8-trimethylpsoralen; UVC = UV radiation at 220–290 nm; MED = minimum erythema dose(s); J = joule(s); mW = milliwatts; MPD_{UVA}, MPD_{blue} = MPD of UVA, MPD of blue radiation; PGE = prostaglandin(s) of the E series; PGF = prostaglandin(s) of the F series.

[1] Presented at the Conference on Photobiologic, Toxicologic, and Pharmacologic Aspects of Psoralens held in Research Triangle Park, North Carolina, March 1–3, 1982.

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skin sensitizing ability suggest a relationship with the ability to cross-link DNA (*1, 2*). A relationship with the extent of singlet oxygen production of psoralens has also been proposed (*3*). In this discussion, phototoxicity due to 8-MOP will be used as a prototypic example unless otherwise specified.

ORAL 8-METHOXYPSORALEN

Following oral administration of 8-MOP, most available preparations cause maximum serum levels between 0.5 and 3 hours (*4*). Considerable differences in peak serum levels have been shown in most studies, in some varying as much as 80-fold following comparable doses on a weight-for-weight basis (*5*), although in other studies variations were less striking (*4, 6, 7*). Differences may be ascribed to individual variations in speed and degree of absorption; varying bioavailability of different preparations may lead to alterations in the size and time of peak serum levels (*6*). Serum 8-MOP levels are a function of the oral dose in a given individual (*7*).

Some correlation between serum levels and the degree of cutaneous photosensitivity has been shown. The degree of cutaneous photosensitivity is assessed by measurement of the amount of UVA irradiation required to induce a standardized degree of skin erythema measured 48–72 hours later: This is the MPD. The peak of photosensitivity is the time at which the MPD is lowest and generally coincides with peak serum levels, which suggests a rapid distribution of the drug into the skin (*4, 8*). Repeated phototesting of 6 volunteers receiving in sequence different doses of 8-MOP showed a good correlation between the MPD and serum levels (*7*); repeated testing of the same individuals diminished the effect of other variables, such as intrinsic sensitivity to UVA, pigmentation, and absorption. In contrast, when isolated determinations of 8-MOP serum levels and MPD were made in different individuals, little correlation between the 2 measurements was demonstrable (*9*).

Although most protocols for oral PUVA therapy indicate that a 2-hour interval between oral psoralen and irradiation is optimal, some individuals may show more delayed peak photosensitivity and peak serum levels (*10*).

Psoralens and 8-MOP are detoxified and excreted as glucuronides and hydroxylated products. The TMP is metabolized principally to 5-carboxy-4,8,-dimethylpsoralen. Because this is not a skin photosensitizer, TMP causes less sensitivity than does 8-MOP when administered orally, although topically it is a potent photosensitizer. Eighty percent of administered psoralens are excreted in the urine in 8 hours (*11*).

TOPICAL PSORALENS

Some experience has been obtained by investigators using topical psoralen solutions to induce photosensitization. Aqueous TMP and 8-MOP have been studied at various concentrations (*12, 13*). When 15-minute dilute aqueous baths (0.3–0.7 mg/liter) are used, maximum sensitivity is present immediately after bathing and declines to about one-quarter of this level after 1 hour. In general, much higher degrees of photosensitivity, i.e., much lower MPD, are obtainable with topical solutions. More concentrated alcoholic solutions applied to the skin result in a more persistent but not necessarily greater photosensitivity, lasting many hours or days.

DOSE-RESPONSE EFFECTS

The MPD is usually defined as the smallest UV dose which causes homogeneous pinkness of the skin filling the irradiation site and possessing clear margins defining the area. It is important for one to standardize and report the time between psoralen ingestion or application and exposure and the time between UV exposure and inspection of the skin. Most MPD are read 48 hours after UV exposure. An increase in the irradiation dose increases the intensity of erythema; marked edema and blistering may result from higher doses. The dose–response curve or increase in the intensity of the response with UVA dose rises more steeply than for UVB and UVA alone, which may also cause edema and blistering at high doses. In contrast, UVC has a much flatter dose–response curve because humans tolerate up to 200 times the MED of UVC with only slight intensification of erythema. We treated a series of subjects with aqueous psoralen (0.003%) and exposed them to graduated doses of UVA irradiation to determine their MPD. Those patients who developed edematous reactions at higher doses did so at an average of 2.7 ± 0.9 times the MPD. Patients who developed blistering reactions did so at an average of 5 ± 0.8 times the MPD. The average MPD of those who blistered at higher doses was 0.36 J/cm^2, which is not significantly different from the average MPD of all subjects in the study. However, it is possible that those volunteers who developed edema or blistering reactions might be a distinctive subgroup. Following oral 8-MOP, blistering developed in 7 of 44 sites exposed to a quadruple, 4 of 44 sites receiving a triple, and 0 of 44 sites receiving a double MPD.

TIME COURSE

Erythema resulting from psoralen phototoxicity shows a longer latency between irradiation and onset than does erythema resulting from UVA, UVB, or UVC irradiation of unsensitized skin. Erythema is also of later onset than the erythema caused by most other sensitizers, such as anthracene or porphyrins, which act through photodynamic pathways and cause photooxidation of biologic substrates. Erythema induced by PUVA may not begin until 24 hours after exposure, when that induced by UVB is at or past its maximum. Maximum erythema from psoralen occurs at 48–72 hours. Intense responses, resulting from 3 to 4 MPD, may begin earlier and be visible at 24 hours and peak later than 48 to 72 hours. Intense responses may be persistent for several weeks, then blend into the hyperpigmentation which also results from PUVA.

Other aspects of the phototoxic responses are also delayed. In the hairless mouse, skin edema due to psoralen phototoxicity begins after a latency of 14 hours, after which skin thickness and water content increase rapidly and reach a plateau at 24–48 hours (*14*). The induction of ornithine

decarboxylase is also delayed for a similar period (*15*), whereas after UVB irradiation in unsensitized skin (*16*) or UVA irradiation in anthracene-treated skin (*15*), ornithine decarboxylase activity is elevated within 4 hours. This event precedes cellular proliferation and may be related to replacement of epidermal cells after phototoxic damage.

ACTION SPECTRUM STUDIES

The absorption maxima of 8-MOP, TMP, and psoralen lie in the region of 210–330 nm. Therefore, it might be expected that wavelengths in these regions would have the greatest photosensitizing effect. However, cutaneous photosensitization action spectra for psoralens indicate that wavelengths longer than 320 nm have the greatest apparent photosensitizing effect. In a study using oral and topical photosensitization in the guinea pig, Nakayama et al. (*17*) demonstrated peak activity at 330 nm. However, comparison of energy requirements for erythema production in untreated and treated animals showed that the MED in the region of 250–290 nm was significantly less in the psoralen-treated animals. Other workers have shown maximum photosensitization to 360 nm (*18, 19*). Investigators conducting 2 recent studies evaluated action spectra for PUVA-induced phototoxicity by measuring sunburn cell formation in the skin and by observing delayed erythema (*20, 21*). Peak activity for the induction of sunburn cells in mouse epidermis was observed in the region of 320–335 nm. At 305 and 312.5, the control animals and 8-MOP-treated animals showed identical responses. In human skin, Cripps and Lowe (*21*) found that maximum photosensitization shown by delayed erythema was 320–335 nm for TMP and 313–320 nm for 8-MOP.

Several explanations may exist for discrepancies between measured action spectra for a biologic response and the absorption maxima of the photosensitizing compound, or chromophore, when it is known. Photobiologic responses occurring in the skin are modified by the optical properties of different skin layers. Inasmuch as penetration of radiation through the skin is dependent on wavelength, the spectral distribution of radiation reaching a given layer varies. The relatively poor penetration of shorter wavelengths through the stratum corneum may at least partly account for their lack of efficacy in the induction of psoralen photosensitization in vivo. However, because the site at which the initial photochemistry occurs in psoralen photosensitization is not known, the contribution of this effect cannot be estimated accurately.

When photosensitization leads to a response which also occurs in the absence of the photosensitizers, e.g., erythema, the action spectrum obtained by plotting 1/MPD against wavelength may not truly identify the principal region where photosensitization is occurring. Two photoproducts resulting from the sensitized and unsensitized pathways may interact additively to induce a response (*22*). Erythema induced by psoralen photosensitization in the skin is a typical example of such a response. For example, with 8-MOP, with an absorption peak at 303 nm, if the effects of skin optics are discounted for the moment, formation of a photoproduct may occur most effectively in this region, i.e., with the minimum energy requirement. However, as this amount of energy is greater than that required to induce erythema by the unsensitized pathway, the effect of the presence of psoralen upon erythema induced by 303-nm radiation would be largely obscured. Although the erythemogenic effects of psoralen extend to longer wavelengths, the relative contribution at 330 nm when compared with the contribution of the unsensitized pathway (330-nm radiation alone) may be much greater. This effect will also be amplified by the greater penetration by longer wavelengths.

For a true action spectrum to be derived, when both sensitized and unsensitized pathways lead to a similar response, a comparison and appropriate mathematical analysis of the responses resulting from sensitized and unsensitized pathways simultaneously are required. However, limitation in accuracy of erythema measurement with current technology would make it difficult for one to detect the small differences in energy requirement at 303 nm for the induction of similar degrees of erythema in topically sensitized and unsensitized skin.

It is speculated but not proved that cutaneous phototoxicity is related to the cross-linking of DNA in the epidermis. Evidence for this is circumstantial and principally revolves around comparative data relating cross-linking ability of different psoralens in vitro with their erythemogenic potential. Different compounds may also vary in other respects, such as penetration, absorption, metabolism, distribution in the body, and the ability to induce the formation of singlet oxygen, which may also be important in phototoxicity. An alternative approach for an investigator to study the relationship between cross-linking ability and phototoxicity is to attempt to favor the generation by 1 psoralen of different photoproducts, e.g., cross-link or monoadducts, by variation in exposure conditions.

Cross-linking of DNA is an event requiring 2 photons: Formation of a 3,4- or 4′5′-monoadduct results from the absorption of a single photon by an intercalated psoralen molecule; absorption of a second photon by a suitably aligned 4′5′-monoadduct leads to further cycloaddition across the 3′4-bond of the pyrone ring of the psoralen to a base in the opposite strand of DNA. A shift in the absorption spectrum of the psoralen molecule toward shorter wavelengths following monoadduct formation results in a relative loss of absorption in the 380- to 400-nm region, where small but significant absorption by intercalated unbound psoralen occurs. Irradiation in this region will therefore cause monoadduct formation but will not be absorbed by the monoadduct so formed; thus further photochemical bonding and cross-link formation will not be favored by irradiation in this region. This has been demonstrated in isolated DNA. Chatterjee and Cantor (*23*) demonstrated differences between the action spectra for monoadduct formation by 4′-aminomethyl-4,5′,8-trimethylpsoralen and for the conversion of monoadducts into cross-links, the latter action spectrum showing a shift toward shorter wavelengths. The difference was exploited in fibroblast cultures (*24*) in studies which showed that the kinetics of growth inhibition induced by either 8-MOP and 395-nm radiation or by a monofunctional psoralen (angelicin) and UVA were similar and were of shorter duration

than the effects of 8-MOP plus UVA, which leads to cross-linking and prolonged growth inhibition. In bacterial systems, 8-MOP and blue light (greater than 380 nm) was used by Grossweiner and Smith (*25*) to induce monoadducts.

Other approaches to the selective generation of monoadducts from bifunctional psoralen include the use of high psoralen concentrations and low irradiation levels, thereby maximizing single-photon photochemistry relative to 2 photon processes. Because cross-linking requires the absorption of 2 consecutive photons, firstly by an intercalated psoralen and secondly by the resultant psoralen–DNA monoadduct, the generation of monoadducts is favored; however, of necessity only low yields can be obtained.

Pulsed laser irradiation has also been used. This approach relies on the finite time required following single-photon absorption before a monoadduct is formed which is in a conformational state capable of absorbing a second photon and becoming converted into a cross-link. The use of a short (10–15 nanoseconds) pulse or a short period between 2 successive pulses favors a predominance of monoadducts (*26*).

One can use split irradiation schedules to favor cross-linking. Cultures have been irradiated first in the presence of psoralen, inducing both monofunctional and bifunctional adducts, followed by re-irradiation in the absence of free psoralen so that cross-linking of existing monoadducts is maximal without causing further monoadduct formation (*27–29*). The use of low psoralen concentrations and high radiation doses should also favor cross-linking because the chances of 2 photon events will be maximized.

We have studied the interaction between UVA and radiation greater than 380 nm (blue light) in cutaneous phototoxicity due to 8-MOP in humans. Our initial studies were based on the following theses:

1) If a certain density of cross-links in epidermal DNA results in erythema, irradiation regimens which are more effective at inducing cross-links will be more erythemogenic (less energy causing the same degree of erythema).

2) Because blue radiation favors monoadduct formation and is inefficient at inducing cross-links, high energies of blue radiation alone will be required to induce enough cross-links to cause erythema.

3) Monoadducts induced by suberythemogenic doses of blue radiation are available for cross-linking by subsequently administered UVA, if given within the time needed for monoadduct repair. Although the relative spectral absorption characteristics of intercalated 8-MOP and 8-MOP–DNA monoadducts should strongly favor monoadduct formation by blue irradiation, potentially high densities of monoadducts could be formed and be available for cross-linking by UVA. Under these conditions small amounts of UVA irradiation could lead to substantial cross-linking and erythema; a synergistic effect between blue and UVA irradiation would result.

4) In contrast, if a suberythemogenic dose of UVA is given *first,* blue irradiation given subsequently will not be effective in cross-linking previously formed monoadducts; at best, blue irradiation given secondly might be additive with respect to erythemogenesis.

5) If monoadducts persist in the skin longer than does free psoralen, initial suberythemogenic irradiation resulting in monoadduct formation should result in prolongation of photosensitivity because these monoadducts will be available for subsequent cross-linking. Re-irradiation with UVA at intervals after the initial 8-MOP and blue irradiation could allow the time for removal of monoadducts (repair) to be measured.

MATERIALS AND METHODS

The skin of 15 normal fair-skinned volunteers was used. We applied 8-MOP as a 0.003% aqueous solution to an 8-cm diameter circle of skin using a soak chamber for 15 minutes. This concentration was selected after several preliminary studies (*13*) because it provided consistent and reproducible photosensitization of an appropriate degree and duration. The mean MPD of UVA in human skin treated in this way is 0.26 J/cm^2 immediately after application. This remains constant for 30 minutes, after which photosensitivity declines and is effectively lost after 6 hours.

Ultraviolet irradiation (320–400 nm).—This was provided by two 36-inch fluorescent PUVA tubes mounted 6 inches apart. Peak emission is at 355 nm (fig. 1A), total output at 1.65 mW/cm^2 between 320 and 380 nm was measured with an IL700 radiometer equipped with a UVA sensor.

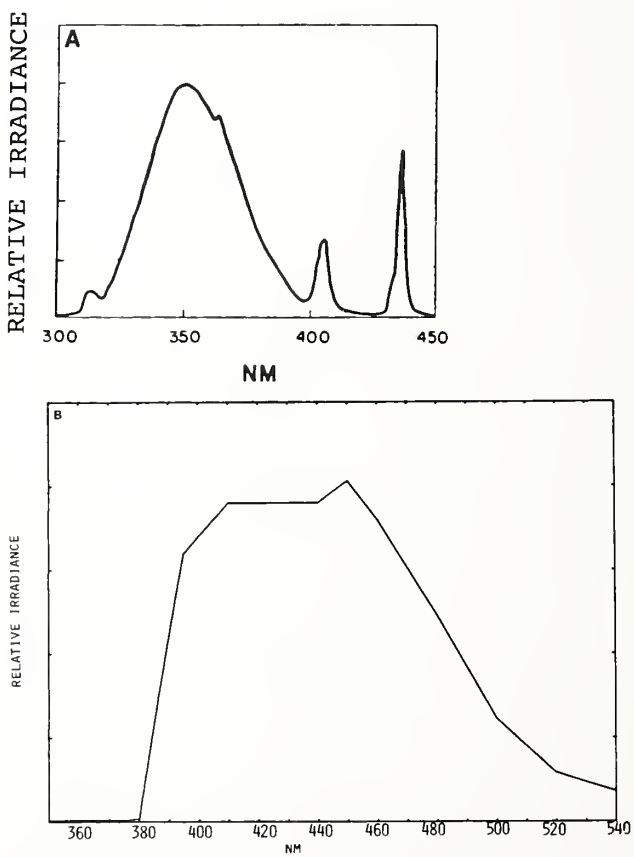

FIGURE 1.—A) Spectral irrradiance of UVA source. B) Spectral irradiance of blue light source.

Blue irradiation.—The output of a 2,500-W xenon arc was filtered through 6 cm of 10% copper sulfate solution and 2.5 cm of 1-64 Lucite, providing a sharp cutoff at 380 nm (fig. 1B); total output was 5 mW/cm^2 between 380 and 400 nm.

Erythema was graded at 48 and 72 hours according to standard criteria:

Grade	Criteria
0	No erythema
Trace	Minimum perceptible erythema
1+	Definite erythema with well-defined margin
2+	Marked erythema (red)
3+	Erythema with edema
4+	Erythema with blistering

Procedure.—Immediately after psoralen application, the MPD of UVA and blue irradiation were determined. A series of 1-cm diameter sites in the soaked area were exposed to graduated radiation doses increasing by 15% increments between adjacent sites. Erythema was graded at 48 and 72 hours, and the MPD taken was the least dose resulting in 1+ erythema.

We then studied the interactions between UVA and blue radiation, using the previously measured MPD_{UVA} and MPD_{blue} to calculate exposure doses equivalent to different fractions of the MPD.

The effect of suberythemal doses of blue radiation on the UVA dose leading to erythema, given before or after blue radiation, was studied. After psoralen application, 2× 8-cm strips of skin received 1/4, 1/8, or 1/16 MPD_{blue}. A series of 8 × 1-cm diameter exposure sites within this area were then exposed to graduated doses of UVA up to 1 MPD_{UVA} in 20% increments.

The order of the procedure was then reversed, with the 8 graduated UVA exposures applied first, followed by exposure of the 8 sites to 1/16, 1/8, or 1/4 MPD_{blue}.

Previous studies demonstrated that during the time required for both irradiations (<30 min) the degree of photosensitivity resulting from psoralen applied in this way does not change.

RESULTS

Minimum Phototoxic Doses

The mean MPD_{blue} was 14.3± 3.1 J/cm^2, and the mean MPD_{UVA} was 0.26± 0.10 J/cm^2 (n= 15); they were read at 48 and 72 hours.

Blue-Ultraviolet Radiation (320-400 nm) Interaction

Areas of skin receiving 0.25 MPD_{blue} required an average of 0.18± 0.08 MPD_{UVA} given subsequently to induce 1+ erythema. If the effects of blue and UVA were simply additive, the sum of the MPD fractions would be 1.0. In practice, the sum of the fractions was 0.25 MPD_{blue} + 0.18 MPD_{UVA} = 0.43.

In areas of skin receiving 0.25 MPD_{blue} *after* graduated doses of UVA were given, 1+ erythema was seen in sites receiving 0.76± 0.17 MPD_{UVA}. This approximates closely with direct photoaddition (0.76 MPD_{UVA} + 0.25 MPD_{blue} = 1.01). Similar differences were present when 1/16 and 1/8 MPD_{blue} were administered before and after UVA irradiation (fig. 2). In each instance when blue was given first, the effects were synergistic; when blue was given second, the result approximated to photoaddition (MPD_{UVA} + MPD_{blue} ~ 1.0, represented by the diagonal line). This order dependence indicates that the photochemistry induced by UVA and blue irradiation differs and that the difference is important with respect to erythemogenesis.

Time Course

The enhanced sensitivity to UVA induced by blue irradiation was studied for 48 hours. Topical psoralen applied in the way described induced photosensitivity which declined rapidly after 30 minutes; at intervals greater than 6 hours after application, sensitivity was effectively and completely lost, i.e., the MPD equaled the MED (fig. 3). After irradiation of an 8-MOP-treated site with 0.25 MPD_{blue}, persistent photosensitivity lasting at least 48 hours was demonstrable, as shown by the ability of UVA to induce erythema at doses less than the MED. In skin not treated with 8-MOP, the average MED= 52.5 J/cm^2. The dose of UVA required to induce 1+ erythema in sites treated 48 hours previously with 8-MOP and 0.25 MPD_{blue} was 0.61± 0.44 J/cm^2.

These results suggest that suberythemogenic doses of blue irradiation, for example, 1/16 MPD_{blue}, cause the formation of a nonerythemogenic photoproduct which persists in the skin for at least 48 hours. This can act as a substrate for subsequent photochemistry induced by UVA irradiation, which induces an erythemogenic photoproduct at exposure doses much less than those required to induce erythema in previously untreated skin. Erythema induced by UVA in such treated areas peaks 48–72 hours after UVA irradiation, following a similar time course to erythema induced by a single UVA irradiation immediately after soaking with 8-MOP solution.

Mediators

Most attempts at identification of the mediators involved in phototoxic erythema due to psoralens have provided negative results. Techniques that have proved successful in identifying mediators involved in UVB- and UVC-induced erythema have been used. Plummer et al. (*30*) administered 3 MPD of UVA to localized areas of nonlesional skin of patients receiving PUVA treatment for psoriasis 2 hours after the oral administration of 8-MOP. Exudate was collected by a suction blister technique from nonirradiated and irradiated areas 24 to 72 hours later. No elevation of PGE_2 or $PGF_{2\alpha}$ was detectable in irradiated compared with nonirradiated areas or with nonirradiated psoriatic skin. Urinary excretion of PGF-M, a major metabolite of PGF_I and $PGF_{2\alpha}$ was also unaffected by a suberythemogenic dose of PUVA applied to the whole body (*31*). These observations suggest that PUVA does not alter the metabolism of arachidonic acid into PG. However, redirection of arachidonic acid metabolism through the lipoxygenase-catalyzed pathways could be occurring.

Studies in vitro provide different results which may not

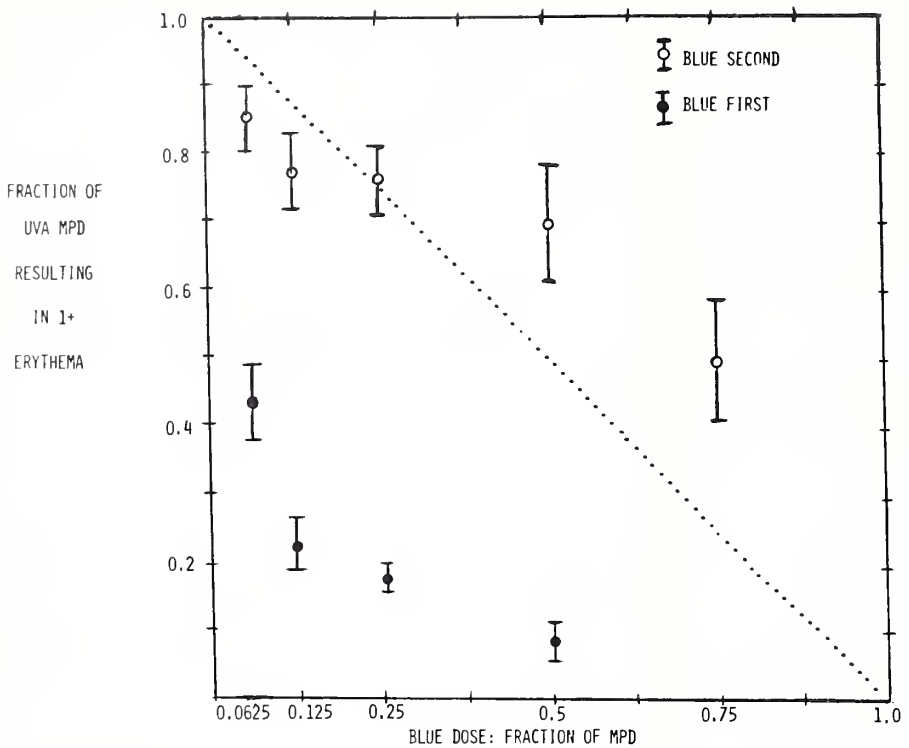

FIGURE 2.—Differences in photosensitivity induced by UVA by blue light: order effect.

be applicable to human skin in vivo. Using rat skin homogenates and subfractions, Lord and co-workers (*32*) found that 8-MOP enhanced the oxygenation of arachidonic acid and transformation to PG. This effect was greater with 254-nm radiation than with 360 nm, corresponding with the absorption maximum for 8-MOP in the UVC region. Without irradiation, 8-MOP stimulated the conversion of PGE_2 to $PGF_{2\alpha}$ resulting in a marked alteration in the ratio of these 2 compounds. The relevance of these observations to cutaneous phototoxicity is not clear at present.

The lack of effectiveness of nonsteroidal anti-inflammatory agents in PUVA erythema, discussed below, also points against a major role for PG in psoralen phototoxicity.

Treatment with PUVA induced a fivefold increase in lipoxygenase activity in the dermis in the hairless mouse (*33*) 24 hours after treatment, thereby supporting a role for lipoxygenase pathway products in PUVA-induced inflammation in the rodent; the activity may be derived from inflammatory cells. No change in the cyclooxygenase pathway was seen.

Eaglestein et al. (*34*) studied the role of neutrophil-derived products in UV-induced erythema in guinea pigs made neutropenic with cyclophosphamide several days before irradiation. This procedure modified the cutaneous response to UVB but not to PUVA, which suggests that a neutrophil product could be important in UVB-induced erythema in the guinea pig but not in that induced by PUVA in this animal.

The role of kinins in PUVA-induced erythema in the

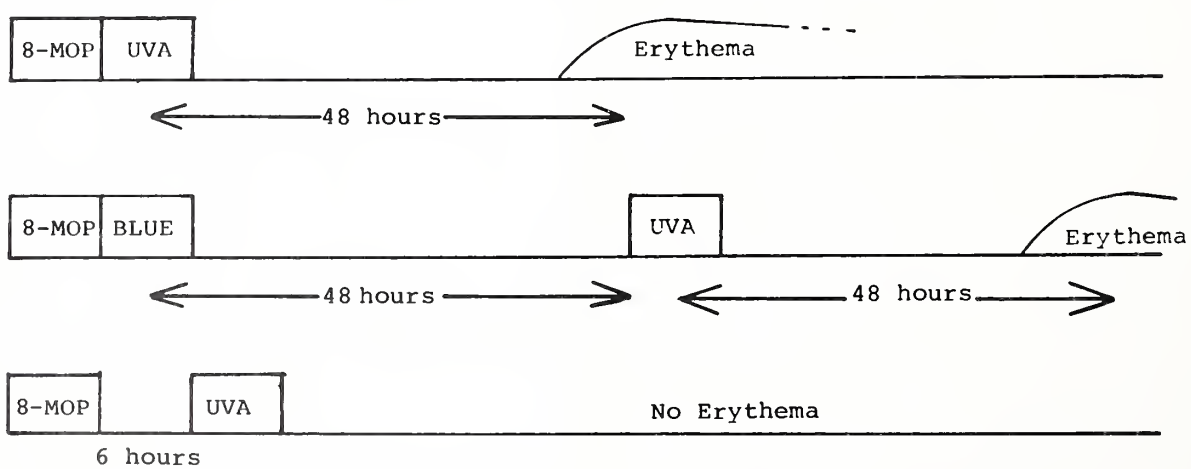

FIGURE 3.—Timing of erythema response in relation to 8-MOP treatment and exposures.

guinea pig was investigated with the use of the kallikrein inhibitor aprotinin. This agent did not alter the erythema response to PUVA. However, bradykinin can be formed by other pathways not inhibited by aprotinin.

Although mast cell degranulation has been observed in skin made erythematous by PUVA in humans (*35*), the lack of effect of antihistamines on PUVA erythema makes a major role for histamine unlikely.

Other anti-inflammatory agents are also generally unsuccessful in modifying the course and intensity of PUVA-induced erythema. The use of corticosteroids systemically does not modify the PUVA erythema response, although evidence indicates that the associated histologic changes may be modified (*36*). We have also been unable to demonstrate an effect of systemic steroids on UVB erythema, in contrast to previous reports (*37*). Betamethasone did not alter PUVA-induced edema in the mouse (*38*).

Indomethacin, either topically or systemically administered, is ineffective in modifying PUVA-induced erythema in man (*39, 40*). Contrastingly, it causes marked reduction of the early phases of erythema due to UVB and UVC. This nonsteroidal anti-inflammatory agent inhibits PG biosynthesis from arachidonic acid.

Indomethacin is highly effective in inhibiting some other manifestations of PUVA-induced phototoxicity in the mouse. The response in this species is characterized by considerable edema beginning 14 hours after irradiation and reaching a maximum of 24 hours. Both topical and ip administration of indomethacin caused considerable dose-dependent inhibition of this response (*14*). An inhibitor of both the cycloxygenase and lipoxygenase pathways of arachidonic acid metabolism, 5,8,11,14-eicosatetraynoic acid also caused inhibition of edema.

Therefore, we have little information about the agents that mediate the striking inflammatory response which follows cell injury in whole skin by PUVA; more studies are needed.

Histopathology

Equally erythemogenic doses of UVA, UVB, UVC, and PUVA (0.6 mg 8-MOP/kg followed by UVA irradiation 2 hr later) were administered to human volunteers and biopsies taken at 1, 2, 3, and 7 days (*41*). Changes induced by PUVA in both epidermis and dermis evolved more slowly than the response induced in nonsensitized skin by UVA, UVB, and UVC. Spongiosis was first observed at 2 days and was maximal from 3 to 7 days. Dyskeratotic cells were less striking than in UVB- and UVC-irradiated sites but were seen between 1 and 7 days. Marked nucleolar enlargement was noted in keratinocytes in the 7-day biopsies only.

In the dermis, a striking infiltration by lymphocytes was present around blood vessels, extending generally through the middle one-third of the dermis; the depth was proportional to the intensity. Vascular damage was first noted 3 days after exposure and was equally intense at 7 days. Endothelial cell swelling, red cell extravasation, and nuclear dust were usually present simultaneously.

A detailed comparison of the effects of topical and orally administered psoralens is not well documented. Konrad et al. (*35*) observed that cellular infiltration was more striking and less predictable relative to dose in topically sensitized individuals. The findings of this study also included mast cell degranulation and were believed to be morphologically identical to those that follow UVB irradiation.

In 4 of 6 patients who received PUVA treatment for psoriasis (*42*), blistering occurred at the site of edema, inflammation, or friction. Necrosis or dissolution of the stratum malpighii was almost always present, particularly in the lower layers; little dermal infiltration was seen. Sunburn cells were frequently observed in adjacent skin, which suggested a relationship between the blistering and phototoxicity.

REFERENCES

(*1*) Dall'Acqua F, Marciani S, Vedaldi D, et al: Studies on the photoreactions (365 nm) between DNA and some methylpsoralens. Biochim Biophys Acta 353:267–273, 1974

(*2*) Musajo L, Rodighiero G, Caporale G, et al: Photoreactions between skin-photosensitizing furocoumarins and nucleic acids. *In* Sunlight and Man: Normal and Abnormal Photobiologic Responses (Fitzpatrick TB, Pathak MA, Harber LC, et al, eds). Tokyo: Univ Tokyo Press, 1974, pp 369–387

(*3*) De Mol NJ, Beijersbergen van Henegouwen GM: Relationship between some photobiological properties of furocoumarin and their extent of singlet oxygen production. Photoehem Photobiol 33:815–819, 1981

(*4*) Swanbeck G, Ehrsson H, Ehrnebo M, et al: Serum concentration and phototoxic effects of methoxsalen in patients with psoriasis. Clin Pharmacol Ther 25:478–480, 1979

(*5*) Thune P, Volden G: Photochemotherapy of psoriasis with relevance to 8-methoxypsoralen plasma level and low intensity radiation. Acta Derm Venereol (Stockh) 57: 351–355, 1977

(*6*) Ljunggren B, Carter DM, Albert J: Plasma levels of 8-methoxypsoralen determined by HPLC in psoriatic patients ingesting drugs from two manufacturers. J Invest Dermatol 74:59–62, 1980

(*7*) Ljunggren B, Bjellerup M, Carter DM: Dose response relations in phototoxicity due to 8-methoxypsoralen and UVA in man. J Invest Dermatol 76:73–75, 1981

(*8*) Thune P: Plasma levels of 8-methoxypsoralen and phototoxicity studies during PUVA treatment of psoriasis with meladinin tablets. Acta Derm Venereol (Stockh) 58: 149–151, 1978

(*9*) Steiner I, Prey F, Gschnait F, et al: Serum levels of 8-methoxypsoralen 2 hours after oral administration. Acta Dermatol Venereol (Stockh) 58:185–188, 1978

(*10*) VanWeelden H, Veen DE, Vries CG, et al: The sensitivity of human skin to UV-A as a function of time after oral administration of 8-MOP. Arch Dermatol Res 268:315–317, 1980

(*11*) Pathak MA, Dall'Acqua F, Rodighiero G, et al: Metabolism of psoralens. J Invest Dermatol 62:347, 1974

(*12*) Fischer T, Alsin J: Treatment of psoriasis with trioxsalen baths and dysprosium lamps. Acta Derm Venereol (Stockh) 56:383–390, 1976

(*13*) Jaenicke KF, Anderson RR, Blank IH, et al: The effects of concentration and vehicle on topical 8-methoxypsoralen induced photosensitization. Presented at the Ninth Annual

Meeting of the American Society for Photobiology, Williamsburg, Va., June 14–19, 1981

(*14*) Cleaver L, Gange RW, Folsom KT: Skin edema due to PUVA in the hairless mouse and effects of antihistamine on indomethacin. J Invest Dermatol 76:322, 1981

(*15*) Gange RW: Epidermal ornithine decarboxylase activity and thymidine incorporation following treatment with ultraviolet A combined with topical 8-methoxypsoralen or anthracene in the hairless mouse. Br J Dermatol 105: 247–255, 1981

(*16*) Lowe NJ, Verma AK, Boutwell RK: Ultraviolet light induces epidermal ornithine decarboxylase activity. J Invest Dermatol 71:417, 1978

(*17*) Nakayama Y, Morikawa F, Fukuda M, et al: Monochromatic radiation and its application: Laboratory studies on the mechanism of erythema and pigmentation induced by psoralens. *In* Sunlight and Man: Normal and Abnormal Photobiologic Responses (Fitzpatrick TB, Pathak MA, Harber LC, et al, eds). Tokyo: Univ Tokyo Press, 1974, pp 591–611

(*18*) Buck HW, Magnus IA, Porter AD: The action spectrum of 8-methoxypsoralen for erythema in human skin. Br J Dermatol 72:249–255, 1960

(*19*) Pathak MA: Mechanisms of psoralen photosensitization and in vivo biological action spectrum of 8-methoxypsoralen. J Invest Dermatol 37:397–407, 1961

(*20*) Young AR, Magnus IA: An action spectrum for 8-MOP induced sunburn cells in mammalian epidermis. Br J Dermatol 104:541–548, 1981

(*21*) Cripps DJ, Lowe NJ: The action spectra of topical psoralen. A reevaluation. J Invest Dermatol 70:214, 1978

(*22*) Hartmann PS: The masking model: A possible explanation for various effects of near UV radiation. Photochem Photobiol 34:39–43, 1981

(*23*) Chatterjee PK, Cantor CR: Photochemical production of psoralen–DNA monoadducts capable of subsequent photo-crosslinking. Nucleic Acids Res 5:3619–3633, 1976

(*24*) Pohl J, Christophers E: Photoinactivation and recovery in skin fibroblasts after formation of mono- and bifunctional adducts by furocoumarin and UVA. J Invest Dermatol 75:305–310, 1980

(*25*) Grossweiner LI, Smith KC: Sensitivity of DNA repair-deficient strains of *E. coli* K12 to various furocoumarins and near ultraviolet radiation. Photochem Photobiol 33:317–323, 1981

(*26*) Johnston BH, Johnson MA, Moore CB, et al: Psoralen–DNA photoreaction: Controlled production of mono- and di- adducts with nanosecond ultraviolet laser pulses. Science 197:906–908, 1977

(*27*) Seki T, Nozu K, Kondo S: Differential causes of mutation and killing in *Escherichia coli* after psoralen and light treatment: Monoadducts and cross-links. Photochem Photobiol 27:19–24, 1978

(*28*) Pohl J, Christophers E: Photoinactivation of skin fibroblasts by fractionated treatment with 8-MOP and UVA. J Invest Dermatol 73:176–179, 1979

(*29*) Kaye J, Smith CA, Hanawalt PC: DNA repair in human cells containing photoadducts of 8-MOP or angelicin. Cancer Res 40:696–702, 1980

(*30*) Plummer NA, Hensby CN, Warin AP, et al: Prostaglandins E_2, $F_{2\alpha}$ and arachidonic acid levels in irradiated and unirradiated skin of psoriatic patients receiving PUVA treatment. Clin Exp Dermatol 3:367–369, 1978

(*31*) Kornhauser A: Molecular aspects of phototoxicity. Ann NY Acad Sci 346:398–418, 1980

(*32*) Lord JT, Ziboh V, Poitier J, et al: The effects of photosensitizers and ultraviolet radiation on the biosynthesis and metabolism of prostaglandins. Br J Dermatol 95:397–406, 1976

(*33*) Ruzicka T, Walter JF, Prinz MP: Altered arachidonic acid (AA) metabolism in UV-irradiated hairless mouse (HM) skin. J Invest Dermatol 78:357, 1982

(*34*) Eaglestein WH, Sakai M, Mizuno N: Ultraviolet radiation induced inflammation and leukocytes. J Invest Dermatol 72:59–63, 1979

(*35*) Konrad K, Gschnait F, Hönigsmann H, et al: UVA-mediated psoralen tissue interaction: Subcellular effects. J Invest Dermatol 66:258, 1976

(*36*) Momtaz K, Harrist TJ, Parrish JA: Effect of systemic corticosteroids on the histologic changes induced by acute PUVA phototoxicity. J Invest Dermatol 78:350, 1982

(*37*) Greenwald JS, Parrish JA, Jaenicke K, et al: Failure of systemically administered corticosteroids to suppress UVB-induced erythema. J Am Acad Dermatol 5:197–202, 1981

(*38*) Ljunggren B, Moller H: Interaction of methotrexate and betamethasone with experimental phototoxic inflammation to PUVA (psoralen and PUVA) in mice. Arch Dermatol Res 264:257–260, 1979

(*39*) Morison WL, Paul BS, Parrish JA: The effects of indomethacin on longwave ultraviolet induced delayed erythema. J Invest Dermatol 68:130–133, 1977

(*40*) Gschnait F, Pehamberger H: Indomethacin does not affect PUVA-induced erythema. Arch Dermatol Res 259:109–111, 1977

(*41*) Rosario R, Mark GJ, Parrish JA, et al: Histologic changes produced in skin by equally erythemogenic doses of UV-A, UV-B, UV-C and UV-A with psoralens. Br J Dermatol 101:299–308, 1979

(*42*) McGibbon DH, Vella Briffa D: Histological features of PUVA-induced bullae. Clin Exp Dermatol 3:371–375, 1978

DISCUSSION

R. Brickl: Dr. Parrish, you said that if you gave blue light before and after UVA you augmented the ability of topically applied psoralens to induce delayed erythema. It might be that, with the blue light, you form the monolinks, and probably once a monolink is formed the quantum yield for cross-linking is much higher than if you have only the low amount of this noncovalent binding. So you have just increased the amount of psoralens that might be reactive.

I think the other interesting point is that with a small amount of topically applied psoralen you get a quick decline in response, in contrast to a longer response with a higher amount of psoralens due to a reservoir of psoralen in the epidermis. This gives rise to this long reactivity because, even though the psoralen in the DNA disappears, you may have a reservoir and possible re-formation of complex.

Concerning the oral situation, we found that plasma levels after oral application decline much more rapidly than does the sensitivity to light. That is, after perhaps 5 to 6 hours, plasma levels are already close to zero, whereas the skin sensitivity is still high. I find this result a little difficult to explain, unless there are any distribution effects. Could you comment on this?

J. A. Parrish: The interpretation that you gave for this observation is the one I find most appealing, and, in fact,

you said it better than I could have said it myself. Thank you.

I cannot explain why the time course for photosensitization is longer than the serum level seems to dictate, unless there are some possibilities of stores in the skin or elsewhere. I really do not know how to explain why the serum level does not always correlate with photosensitivity. Possibly the cell level is not always exactly comparable with the serum level. I do not have a good explanation for that observation.

L. Grossweiner: I want to comment that a number of workers have correlated the skin photosensitization with the cross-linking of furocoumarins, which would be consistent with the explanation you have just given. However, I think one cannot rule out the possibility (I am not necessarily supporting this, but it is a possibility) that the monoadducts are efficient generators of singlet oxygen. Some in vitro evidence confirms this characteristic. This would have to be cleared to explain the results of Dr. Parrish on the basis of cross-linking.

J. Hearst: I would like to develop this idea a little further. I think the position that blue light creates no cross-links is an overstatement. We found that with in vitro DNA, the best we could do was enhance the monoaddition over cross-linking by a factor of 2 or 3 by using the longer wavelength. On the other hand, I think that is good enough. I believe that the point you are presenting is identical to the idea that Dr. Hanawalt presented: You can do the same thing with time that you can do with the wavelengths of light; if, in fact, you irradiate skin for a short period initially and then let the psoralen clear from the skin, with the monoadduct left behind, and then do a subsequent irradiation, the consequence should be identical to the blue light treatment.

K. Smith: I think I have mentioned this before at similar meetings because we must constantly keep it in mind: All scientific studies have to be remembered with regard to the precise wavelengths of light being used. The clinician should not rush into the clinic with the scientific laboratory studies until the light is matched with the drug.

The observation is that some of the psoralens have a different absorption spectrum before they combine chemically to form the different monoadducts. Once the compounds have made a monoadduct, their absorption spectra are shifted. This was originally described by Professor Rodighiero.

So I think we have to remember that all the different wavelengths of light have to be considered. For example, the blue light will produce both kinds of monoadducts but only one kind of cross-link because 1 of the monoadducts does not absorb in the blue wavelength. One has to keep these kinds of things constantly in mind to understand the types of reactions that can be expected with the different light sources.

D. M. Carter: Is it possible to remove the erythema-producing properties of these psoralen compounds while maintaining their therapeutic efficacy? Could you attempt to reconcile the points about the effects of 1) oxygen in producing redness, 2) monoadducts in producing redness, and 3) all when one deals with a cross-linking compound using the various light sources we referred to previously?

Several factors are operative at the same time. Clinically, it is difficult to separate these factors in a biologic sense when one bases the comparison on chemical properties.

Parrish: I will try to remember those points; I think that we do not know how the erythema reaction relates to clinical clearing. By that I mean, I do not know whether erythema has to happen, if the therapeutic lesion and the lesion leading to inflammation are the same.

The point that Dr. Hearst was making is a good one. I do not mean to overstate the wavelength component of this argument. It is real, but it should not be overstated because you can do the same thing with split-dose UVA, as Dr. Hanawalt has shown. This kind of thinking is certainly not original with us; it has been done in tissue cultures of bacteria in test tubes by many people in this room. However, if you use split-dose UVA, you see the same phenomena. If you give a small dose early, then photosensitization does not go away in 2 or 3 hours; it remains for many hours. That effect is not as impressive as if you use the wavelength argument plus the UVA. If you use blue light, the effect is more impressive than it is with UVA.

As far as what photoproducts lead to inflammation, I think probably many do. I believe inflammation is a nonspecific response to cell injury, and cells with photons can be injured in many ways. You can also injure membranes, DNA, protein, etc. If you injure the cell in enough ways to cause it to start to fall apart and its mechanisms shut down, the wrenches in various places in the machine release chemicals that act as mediators of information.

I think it is going to be most frustrating to say which chromophore causes inflammation. I think there are many: Some are oxygen dependent, some are not; some are DNA, some are not. I have no idea which photoproduct leads to therapeutic effects.

Carter: It depends on which therapeutic effect you are talking about, too.

Parrish: Right.

Carter: I am considering one in psoriasis and one in hyperpigmentation.

Parrish: I suspect the therapeutic mechanism does differ quite a bit from disease to disease. Also, one thing to be careful of is not to overinterpret the cuff around the arm causing anoxia. This is relative hypoxia and certainly not absence of oxygen in tissue because oxygen is certainly present. This work was done by Dr. Gange and Dr. Anderson in my laboratory.

G. Lazarus: I am having trouble with your definition of inflammation. You told us that the primary site of damage, at least to some degree in prolonged problems, is the dermis. More than likely, in view of what you have presented and the information in the literature, it is the endothelial cells which are altered in some way, either with DNA photoproducts or by long-term alterations in their metabolism, perhaps in PG synthesis.

This leads me to an open-ended question. What is known about the effects of PUVA or UVA on epithelial cells? Could this be explained by the induction of synthesis of unique new mediators which induce vasodilation and which could be studied biologically and biochemically in test animals?

Parrish: I certainly think that long-term effects from inflammation probably do involve some direct photon effect on vessels. There again, I believe we are so tempted to make the story simple, but it is a most complicated one. I suspect by the time you see enough cell injury to induce inflammation that part of the injury is due to keratinocyte damage leading to vessel alterations; part is due to damage at various sites in the dermis leading to inflammation, and part is also due to direct effect on vessels.

I suspect the photons clearly do reach endothelial cells and should alter and damage them. Whether you need mediators or either UVA or PUVA is not clear; we just do not have any information. It would be a good area to work in because the nonunique mediators, i.e., the UVB-induced mediators, do not seem to be doing much. It is quite possible that other types of mediators need to be researched.

Lazarus: The state of endothelial cell biology is undoubtedly far advanced, and we resolved the kinds of problems which were almost insurmountable in the production of endothelial cell cultures. Endothelial cells have unique and specific markers, and some investigators are now rescuing various messages. Genes are cloned which are related to induction to proinflammatory moieties. It seems to me that is an exceedingly fruitful area to study because it would explain erythema at least.

Parrish: I totally agree. That would be a fruitful place to look, in vitro, at the effect on endothelial cells. It also may be more exciting than simply explaining some components of inflammation because perhaps some of the therapeutic effects in psoriasis result from direct vessel effects.

I think we are placing too much emphasis on the effects on DNA in the epidermis. For therapeutic effects of psoriasis, it may not be primarily DNA and it may not be primarily in the epidermis. I think that would be an exciting area for research, and I hope somebody does it.

T. Fitzpatrick: I would like to remind you about Dr. Pathak's study that has already been published, i.e., one effect of a monofunctional adduct is to produce cancers in animals with UVA without developing any erythema. In other words, these animals do not have phototoxicity following topical application and exposure to UVA. The results in psoriasis are less impressive and not as reproducible, but they are real. No one doubts that you can cause resolution of psoriasis without using topical 5-methylangelicin or one of the isopsoralens and UVA without producing erythema.

M. Pathak: I do have lots of data on the effects of monofunctional adducts.

K. Kraemer: I was impressed with Dr. Parrish's talk of the delayed erythema in the PUVA patients. There may be an analogy to patients who have defective DNA repair. Patients with xeroderma pigmentosum exhibit delayed erythema from sunlight. They have deficient DNA repair, and the sunlight-induced DNA lesions persist for a long time. The onset of redness is delayed but the redness persists for a much longer time. Persistent DNA adducts may be related to delayed erythema in patients with xeroderma pigmentosum and those treated with PUVA. In the former group, thymine dimers persist and in the PUVA patients psoralen–DNA adducts persist.

I have a question regarding wavelength effects and action spectra. Dr. Song and others showed that the psoralen absorption spectrum is at a shorter wavelength than is the erythema action spectrum. I wonder if some of the panelists might comment on this apparent anomaly in that the action spectrum of the PUVA effect on humans and on animals is different from the absorption spectrum.

R. Brickl: First of all, one basic misunderstanding about the older literature seems apparent. I think sometimes previous investigators only claimed that a quantum yield was better, let us say, at 360 than at 320 nm or so. I think you have to consider what quantum yield means. Quantum yield means effect per quantum of absorbed light; it does not mean effect per quantum irradiated. As the absorption spectrum is already far down at 360 nm, even if the quantum yield were higher, the action spectrum would still be lower at 360 nm. According to our experiments on oral and topical treatment, the maximum efficacy probably would be closer to 320 than to 360 nm.

On the other hand, I think it is not only a question of maximum efficacy irradiation but probably also a question of safety. The discussion should probably start at whether 320 nm would be regarded more safe or less safe than 360 nm because the penetration depth of light also differs with wavelengths. These are interesting questions, but I do not know whether any one has answers to them.

Mutagenic Effects of Psoralens in Yeast and V79 Chinese Hamster Cells[1, 2]

Dietrich Averbeck,[3] Dora Papadopoulo,[3] *and* Ileana Quinto[3, 4, 5]

ABSTRACT—The mutagenic effects of monofunctional and bifunctional furocoumarins (psoralens) plus 365 nm radiation were analyzed in the yeast *Saccharomyces cerevisiae*. Per unit dose of 365-nm radiation, bifunctional compounds were more effective than were the monofunctional for the induction of reverse and forward mutations. The same was observed for the induction of 6-thioguanine-resistant mutants in V79 Chinese hamster cells when we compared the activity of 8-methoxypsoralen and 3-carbethoxypsoralen. An analysis of the kinetics of mutation induction indicated that DNA interstrand cross-links induced by bifunctional psoralens are more prone to error than are monoadducts induced by monofunctional psoralens. In yeast, 8-methoxypsoralen and 4,5′-dimethylangelicin were shown to photoinduce mitotic and nondisjunction. The implications of these findings for the use of psoralens in photochemotherapy and cosmetics are discussed. — Natl Cancer Inst Monogr 66: 127–136, 1984.

Mutagenicity studies on psoralens (furocoumarins) in prokaryotic and eukaryotic cells have received increasing interest in recent years (*1, 2*). One reason for this is the use of psoralens in photochemotherapy and cosmetics (*3*) and the resultant concern about possible mutagenic and carcinogenic hazards (*4–6*). Another reason is the fact that psoralens are effective tools in fundamental research on the relationship between lesions in DNA, their repairability, and the induction of genetic and carcinogenic effects (*7, 8*).

The dark interactions and photochemical reactions of psoralens with cellular macromolecules, especially with nucleic acids, are described well in (*9–11*). So-called bifunctional furocoumarins, such as TMP, 8-MOP, and 5-MOP, photoinduce C_4-cycloadditions to pyrimidines in nucleic acids forming monoadducts and biadducts (cross-links) in DNA (*9*). So-called monofunctional furocoumarins, such as the angelicin 4,5′-DMA, and 3-CP only induce monoadducts in DNA (*12*).

To determine the genetic consequences of the 2 types of photoadducts, we used monofunctional and bifunctional furocoumarins. The yeast *Saccharomyces cerevisiae*, a unicellular eukaryotic organism, has already proved useful for such studies (*2, 7, 8*) because several genetic end points can be investigated. Furthermore, a number of DNA repair-deficient mutants are known that define different repair pathways and facilitate the biochemical analysis of repair processes (*13*).

Here we describe the effects of monofunctional and bifunctional furocoumarins in the presence of 365-nm radiation on survival, induction of cytoplasmic petite mutants (reflecting damage to mitochondrial DNA), and the induction of nuclear genetic events, such as reverse and forward mutation and mitotic nondisjunction (aneuploidy) in yeast. To our knowledge to date, the induction of mitotic nondisjunction after treatment with psoralens and 365-nm radiation has not been reported. This study focuses on furocoumarins of photochemotherapeutic interest. We demonstrate that both types of furocoumarins are mutagenic in the presence of 365-nm radiation. However, the 2 types of psoralens differ in their kinetics of induction depending on the genetic end point considered. Photoadditions by 8-MOP (bifunctional) show a higher effectiveness than do those by 3-CP (monofunctional) for the induction of reverse and forward mutations in yeast. The same is demonstrated for the induction of 6-TG^r mutants in V79 Chinese hamster cells.

An analysis of the mutagenic activity of monofunctional and bifunctional psoralens suggests that certain monofunctional furocoumarins like 3-CP may represent a lower mutagenic and carcinogenic potential than the bifunctional compounds in actual therapeutic use.

MATERIALS AND METHODS

Strains of yeast.—We used the haploid strain N123 (a, *his* 1) (*14*) and a derived clone N123 can^s for studies on survival, induction of cytoplasmic petite mutants, and induction of reversions from histidine dependence to nondependence, as well as the induction of forward mutations from can^s to can^r (*8, 15*). The diploid yeast strain D_7 (Zimmermann) was used for the detection of *ilv*$^+$ revertants as described in (*16*). The induction of mitotic aneuploidy was determined in a diploid yeast strain D_6, which is especially designed for this purpose (*17–19*).

ABBREVIATIONS: TMP = 4,5′,8-trimethylpsoralen; 8- or 5-MOP = 8- or 5-methoxypsoralen; 4,5′-DMA = 4,5′-dimethylangelicin; 3-CP = 3-carbethoxypsoralen; 6-TG^r = 6-thioguanine-resistant; can^s = canavanine sensitive; can^r = canavanine resistant; kJ = kilojoules.

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Media and culture conditions.—The media and culture conditions for the haploid and diploid strains were as described in (*15, 16*). Survival, i.e., the colony-forming ability, was measured on complete growth medium containing 1% yeast extract, 2% bactopeptone, 2% glucose, and 2.6% bacto-agar, all from Difco Laboratories (Detroit, Mich.). The induction of *his*$^+$ revertants in the strain N123 was detected on a minimal medium containing 0.67% bacto-yeast nitrogen base without amino acids (Difco). Forward mutations in the *can1* locus were detected on a minimal medium supplemented with 20 μg histidine/ml and 40 μg L-canavanine-sulfate/ml from Sigma Chemical Company (St. Louis, Mo.). For the detection of induced mitotic aneuploidy in the diploid strain D_6, we followed the protocol established by Parry et al. (*17, 18*). In this strain carrying several genetic markers (*17, 18*), an induced loss of 1 copy of chromosome 7 results in the production of monosomic colonies (2n−1), which are white and resistant to cycloheximide because of unmasking of *ade-3* and cyh^r_2 genes. Thus monosomic colonies were scored on solid complete medium containing 2 mg/liter cycloheximide (Sigma). Survivors were determined with the use of complete medium without the cycloheximide addition.

Cytoplasmic petite mutants, i.e., respiratory-deficient mutants, were detected by the tetrazolium overlay technique (*20*). The colonies were counted after up to 6 days of incubation at 30° C.

Furocoumarins.—The 8-MOP used was a commercial product obtained from Chinoin, S.p.A. (Milan, Italy). Synthetic angelicin and 4,5′-DMA, as well as 5-MOP and TMP, were obtained from Professor F. Dall'Acqua of the University of Padua (Padua, Italy), and Dr. M. A. Pathak of Massachusetts General Hospital (Boston, Mass.), respectively. Chromatographically pure 3-CP was newly synthesized by Dr. E. Bisagni of the Institut Curie [Orsay, France (*21*)].

Photosensitizing treatments.—Exposures to furocoumarins plus 365-nm radiation were performed as described in (*15*). After preincubation in the dark in the presence of the compounds (usually at the concentration of 50 μM if not stated otherwise), the yeast cells in stationary phase of growth were exposed to 365-nm radiation emitted from an HPW 125 Philips lamp [*see* (*22*) for the radiation device] at a dose rate of 1.2 $kJ \cdot m^{-2} \cdot minute^{-1}$ as determined by a digital dosimeter from Ultraviolet Products, Inc. (San Gabriel, Calif.).

After treatment, suitably diluted aliquots of the cell suspension were plated on complete and selective media for the detection of lethal and genetic effects. Experiments were performed at least twice. The figures illustrate data taken from at least 1 of 2 independent representative experiments. The genetic activity of psoralens in the absence of 365 nm radiation or the activity of 365 nm radiation alone was always negligible for the genetic end points studied.

For treatment of the strain D_6, a slightly different protocol was adopted. We treated the cells in the early stationary phase of their growth, washed, and then left them for 48 hours for growth in liquid complete medium to obtain the expression of mitotic nondisjunction, then plated them on solid complete and selective media (*17–19*).

Treatment of mammalian cells in culture.—We used V79 Chinese hamster cells cultivated in Eagle's Minimal Essential Medium supplemented with 10% fetal calf serum obtained from GIBCO (France). These cells, seeded 24 hours at 2.10^5 cells/5 ml of culture medium in 25-cm^2 Falcon vessels, were incubated in the presence of the furocoumarin in phosphate buffer (pH 7.2) for 10 minutes. The cells (in exponential phase of growth) were then exposed to 365 nm radiation. For survival studies, the cells were trypsinized immediately after treatment and counted. Suitable aliquots were seeded so that we obtained 50 to 150 colonies/petri dish. After 7 days of incubation at 37° C, the colonies were fixed, stained, and counted.

For the determination of induced 6-TG^r cells (a forward mutation), we used a slightly modified form of the method described in (*23*). We incubated the treated cells for 4 to 5 days in culture medium, trypsinized, counted, and reseeded them at 10^2 cells/petri dish to determine the cloning efficiency; they were re-seeded in 30 to 40 petri dishes at 10^5 cells/dish for the mutagenicity test. The selective agent, 6-TG, was added at a final concentration of 30 μg/ml at different times, ranging from 5½ to 8 days. Several times of expression were used for each treatment dose for determination of the mutant yield at maximum expression times. The culture medium was renewed on the 8th day, and the cells were fixed and stained on the 15th day after treatment. Colony growth per 5 to 8 petri dishes per dose and per expression time were counted, and the mutation frequencies were determined.

RESULTS

Cell Survival and Induction of Cytoplasmic Petite Mutants in Haploid Yeast

Inasmuch as the furocoumarins used in this study differed greatly in their solubility in water (*11, 12*) and experiments are usually performed at high concentrations, we determined the effects of psoralens on cell survival and the induction of cytoplasmic petite mutants at an equimolar concentration of 5 μM at which maximum solubility can be assumed.

Figure 1 illustrates the survival curves obtained in haploid yeast after treatment with the 3 bifunctional furocoumarins, TMP, 5- and 8-MOP, as well as with the 2 monofunctional furocoumarins, 3-CP and 4,5′-DMA, plus 365-nm radiation.

The LD37 doses in kilojoules/square meter were: 0.35 for TMP, 1.7 for 5-MOP, 3.2 for 8-MOP, 14.4 for 4,5′-DMA, and 18.7 for 3-CP. Although the capacity of the psoralen derivatives for cell killing decreased in similar order to that reported for treatments at high concentrations, e.g., 50 μM (*15*), the differences in LD37 values are not the same, probably due to differences in solubility at these concentrations. To produce the same decrease in survival, we found higher doses of 365-nm radiation were necessary with the monofunctional than with the bifunctional furocoumarins.

Previously, 4,5′-DMA and 3-CP were shown to photobind to DNA about 2 and 2.25 times more efficiently than did 8-MOP, respectively (*8*). Thus the decrease in cell

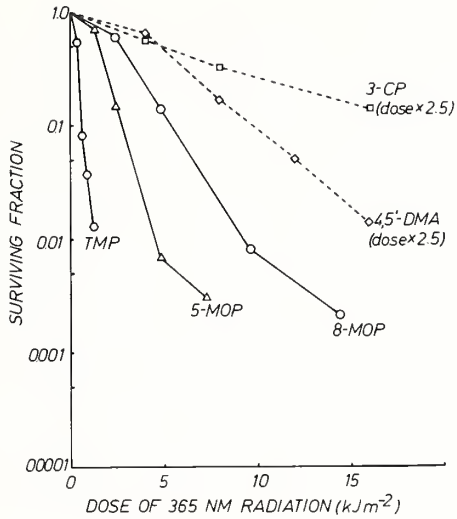

FIGURE 1.—Survival curves obtained for the haploid strain N123 of *S. cerevisiae* after treatment with low equimolar concentrations (5 μM) of TMP, 5-MOP, 8-MOP, 4,5'-DMA, and 3-CP plus 365 nm radiation. For 4,5'-DMA and 3-CP, the indicated doses have to be multiplied by a factor of 2.5. Figure is reproduced with the permission of the publisher of the *Proceedings of the Japanese Society for Investigative Dermatology*.

survival observed after treatments with 8-MOP depended not only on the photobinding capacity but also on the different types of lesions, i.e., the DNA interstrand cross-links induced in addition to the DNA monoadducts. This explanation is likely true also for the strong decrease in cell survival produced by TMP or 5-MOP and 365-nm radiation.

As shown in several cellular systems (*24*) including bacteria (*25*), yeast (*8, 26*), and mammalian cells (*27–29*), this type of lesion is less easily repairable than are the photoinduced monoadducts in DNA.

In yeast, survival data obtained with mutants deficient in DNA repair indicated that different types of DNA repair systems are involved in the repair of damage induced by bifunctional furocoumarins (*30–33*). Excision and error-prone repair pathways as well as repair pathways for DNA double-strand breaks (*30, 33, 34*) and repair steps controlled by *pso*-2 genes (*32*) were shown to take part in the repair of DNA cross-links in yeast. The repair of furocoumarin-induced lesions depended on ploidy, growth phase, and stage in the cell cycle (*35, 36*). Increased repair of 8-MOP plus radiation-induced lesions conferring increased cell survival were seen when low dose rates (365 nm) were used (*8*).

The fate of 8-MOP-photoinduced cross-links in nuclear and mitochondrial yeast DNA was recently analyzed (*26*). For wild-type cells treated in exponential growth phase (10% survival), a removal of 8-MOP-induced cross-links was apparent within 2 hours of posttreatment incubation (*26*), whereas this was not seen in cells treated in the stationary growth phase. The incision of cross-links was inhibited in *rad* 3-2 mutants known to be defective in the excision of 254-nm UV-induced pyrimidine dimers. These authors (*26*) found that the rejoining of single- and double-strand breaks in the course of cross-link repair depended on *rad*-51 and *pso*-2 gene products. In contrast to the repair of cross-links in *Escherichia coli* (*25*), double-strand breaks are intermediates in the overall repair process.

Induction of Cytoplasmic Petite Mutants in Yeast

Figure 2 depicts the induction of cytoplasmic petite mutants in the haploid yeast strain N123 after treatments with monofunctional and bifunctional furocoumarins at low equimolar concentration (5 μM). Per unit of 365-nm radiation dose, the following order of activity was observed for the different psoralen derivatives: TMP > 5-MOP > 3-CP > 8-MOP ≈ 4,5'-DMA (fig. 2A). Because excision repair appears absent or hampered in mitochondrial yeast DNA for DNA cross-links as well as for 254-nm UV-induced pyrimidine dimers (*26*), it is understandable that the data on photobinding were in accord with the pattern of the induction of cytoplasmic petite mutants (*15*).

Apparently, more mutants were produced by the mono-

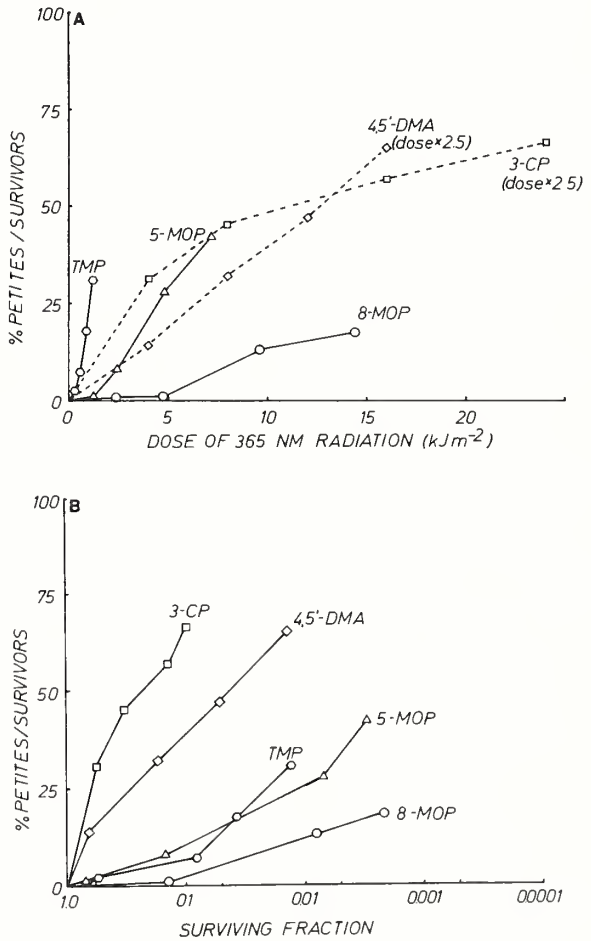

FIGURE 2.—Induction of cytoplasmic petite mutants in the haploid strain N123 of *S. cerevisiae* after treatments with low equimolar concentrations (5 μM) of TMP, 5-MOP, 8-MOP, 4,5'-DMA, and 3-CP plus 365 nm radiation as a function of radiation dose (A), and as a function of survival (B). For 4,5'-DMA and 3-CP, the indicated doses have to be multiplied by a factor of 2.5.

functional than by the bifunctional compounds as a function of survival (fig. 2B). This seems to be related to the differential repairability for lesions induced in nuclear DNA compared with those induced in mitochondrial DNA, as well as to the different effectiveness of the furocoumarins to bind to nuclear and to mitochondrial DNA (*8, 15*). Interestingly, a higher proportion of cross-links was induced by 8-MOP and 365 nm radiation in the adenine–thymine-rich mitochondrial DNA than in the nuclear DNA of yeast (*26*). Psoralens share with other antipsoriatic drugs the property of being effective on the induction of cytoplasmic petite mutants in yeast (*2, 8, 15, 37*).

Induction of Nuclear Mutations in Haploid Yeast

We studied the induction of reversions from histidine dependence to independence and the induction of forward mutations from canavanine sensitivity to canavanine resistance according to a protocol (*8, 15*). Figure 3 shows the induction kinetics as a function of 365 nm radiation for treatments with TMP, 5-MOP, 8-MOP, 4,5′-DMA, and 3-CP with a log/log plot (mutant frequency vs. dose). As observed in (*8, 15*), the activity of the different psoralens follows the order TMP > 5-MOP > 8-MOP > 4,5′-DMA > 3-CP, which is different from the order observed for the induction of cytoplasmic petite mutants. The induction due to 365 nm radiation alone is negligible even at extremely high doses.

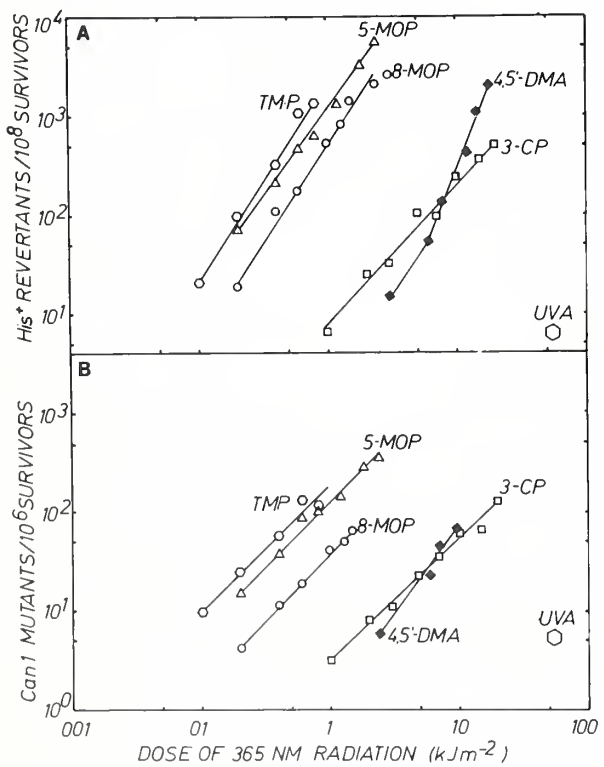

FIGURE 3.—Kinetics of mutation induction in haploid yeast (N123) can^s) after treatment with equimolar concentrations (50 μM) of TMP, 5-MOP, 8-MOP, 4,5′-DMA, and 3-CP plus 365 nm radiation. A) Frequency of induced *his*$^+$ revertants; B) frequency of induced *can* 1-resistant mutants as a function of 365 nm radiation with log/log plots. Induced frequency of mutants due to 365 nm radiation alone is indicated for doses exceeding those used in combination with furocoumarins. Figure is reproduced with the permission of the publisher of the *Proceedings of the Japanese Society for Investigative Dermatology.*

Induction of reversions by the bifunctional furocoumarins TMP, 5-MOP, and 8-MOP follows two-hit kinetics (fig. 3A), whereas the monofunctional compounds 4,5′-DMA and 3-CP exhibit near to one-hit kinetics and one- to three-hit kinetics, respectively. Induction of forward mutants by bifunctional and monofunctional furocoumarins always appears to follow one-hit kinetics with the possible exception of 4,5′-DMA, which deviates slightly. The angelicin-induced reversions (*38*) and the forward mutations (Averbeck D: Unpublished data) follow one-hit kinetics. Forward mutations, which are of the deletion type, are induced with the same kinetics by both types of psoralens.

In contrast, bifunctional psoralens exhibit two-hit kinetics (*7, 8, 15, 38, 39*) for the induction of reversions (fig. 3A). With psoralen (*38*) and 8-MOP (*39*), mutants deficient in excision repair (*rad* 3–12) or in a *pso*-2-dependent repair step concerning DNA interstrand cross-links (*26*) exerted one-hit kinetics for the induction of reversions. This was true at least in the low-dose range. Because one-hit kinetics were found with both strains after angelicin (*38*) and 3-CP photoaddition (*39*), the investigators concluded that in *pso*-2–1 and excision defective strains, monoadducts are mutagenic; however, cross-links induced may exist as non-premutagenic lesions that may essentially lead to lethality (*39*).

Interestingly, the induction kinetics for reversions induced by 8-MOP and 365-nm radiation were reported to be dependent on the dose rate of 365 nm radiation used: One-hit kinetics were obtained at low dose rates, probably due to the absence of cross-links under those conditions (*8*). Furthermore, a recent detailed analysis of the induction kinetics (Averback D: Unpublished results), according to Eckardt and Haynes (*40*) and Kunz et al. (*41*), supported the notion that in the reversion system, a repair system prone to error is induced by the bifunctional furocoumarins 8-MOP and TMP.

When the mutation data were analyzed as a function of survival (*2, 7, 8, 15, 42*), monofunctional compounds, such as angelicin, 3-cyanopsoralen, 3-carbomethoxypsoralen, 3-CP, 5,7-dimethoxycoumarin, and 4,5′-DMA were less efficient for the induction of reversions than were the bifunctional compounds TMP, 5-MOP, 8-MOP, and the metabolite 4,8-dimethyl-5′-carboxypsoralen. This analysis supports the idea (*42*) that the interstrand cross-links induced in DNA by bifunctional furocoumarins may be associated with the induction of error-prone repair.

The results on forward mutations [fig. 3B; (*8, 15*)] suggest differences in the mutagenic capacity depending on the monofunctional furocoumarin used. Furthermore, per viable cell, angelicin was as mutagenic as the bifunctional furocoumarins, and 4,5′-DMA approached the activity of 8-MOP. The 3-CP was less mutagenic than were the bifunctional compounds TMP, 5-MOP, and 8-MOP (*8,*

15). These results call for some caution in the use of certain angular photoreactive furocoumarins (*15*).

Oxygen-dependent Modification of the Genetic Activity of 3-Carbethoxypsoralen

Recently, we (*7, 8, 15, 43, 44*) reported that survival of haploid yeast after treatment with 3-CP was sensitized more efficiently in the presence than in the absence of oxygen. The oxygen-dependent photosensitization by 3-CP appeared related to the high triplet quantum yield of the photosensitizer 3-CP, which was 0.35 in water, whereas for 8-MOP the quantum yield was 0.06 (*44*). Experiments performed with D_2O favored the involvement of singlet oxygen in the photodynamic action of 3-CP (*44*). Because photodynamic photosensitization may produce damage also in cell constituents other than nucleic acids (*45*), we measured the genetic effects induced by 3-CP in the absence and presence of oxygen.

Figure 4 shows the results on the lethal effects and the induction of *ilv*$^+$ revertants in the diploid yeast strain D_7. We noticed a strong photosensitization on both biologic end points in the presence of oxygen and a relatively minor photosensitizing effect in hypoxic conditions. Comparable

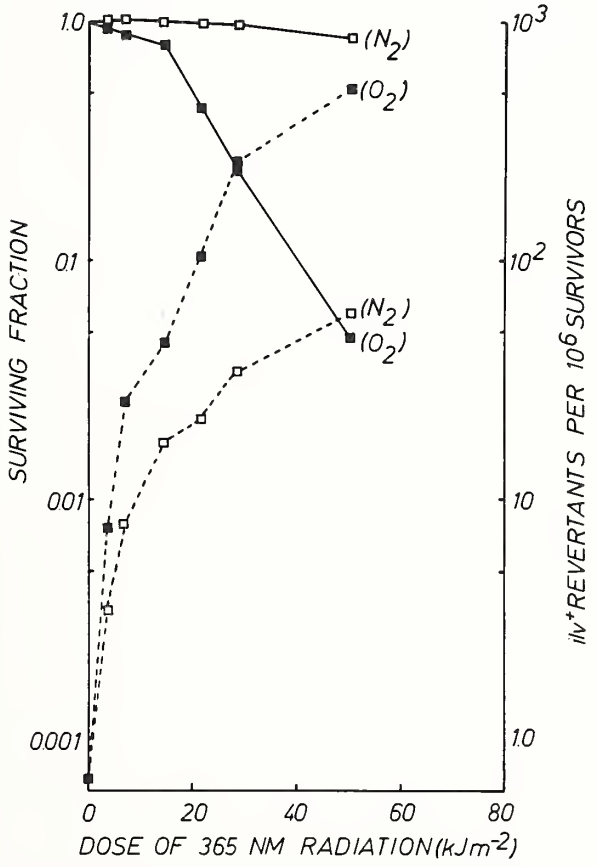

FIGURE 4.—Induction of lethal (*solid lines*) and mutagenic (*dotted lines*) effects in diploid yeast (strain D_7) after treatment at equimolar concentrations (50 μM) of 3-CP with 365 nm radiation under continuous bubbling with pure nitrogen (99.99%) (*open square*) or with air (20% O_2, 80% N_2). The spontaneous mutation frequency is indicated at zero dose.

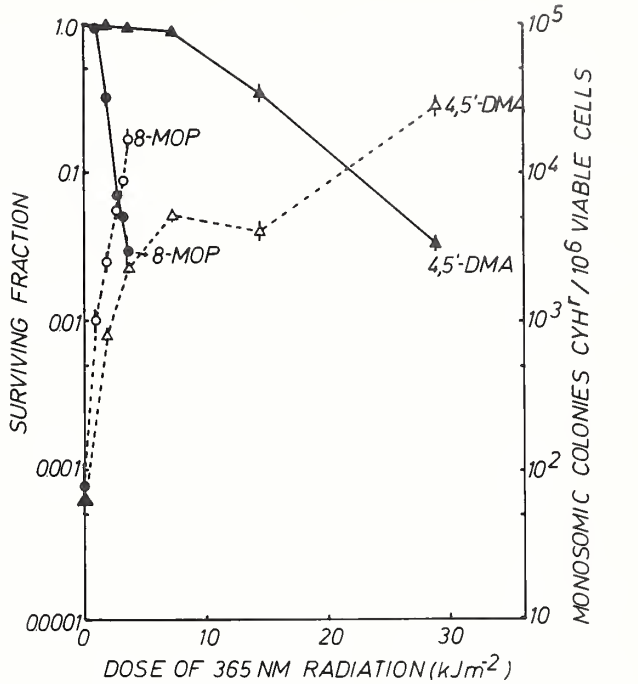

FIGURE 5.—Induction of mitotic nondisjunction as measured by the induction of monosomic cycloheximide-resistant cells (2n-1) in the diploid strain D_6 of *S. cerevisiae* after treatment with equimolar concentrations (50 μM) of 8-MOP and 4,5'-DMA plus 365 nm radiation. Survival data (*solid lines, closed symbols*). Induction of monosomic cycloheximide-resistant cells (*dotted lines, open symbols*). Treatment with 8-MOP (*circles*), 4,5'-DMA (*triangles*). Spontaneous frequency of monosomic cycloheximide-resistant cells is indicated at zero dose.

results were obtained for the induction of mitotic gene conversion (data not shown).

Inasmuch as the induction of genetic effects per viable cell did not differ under the two experimental conditions, it is unlikely that the photosensitization involved differential effects on survival, e.g., high toxicity due to cell lysis caused by membrane damage and mutation induction. Therefore, under both conditions, damage to DNA appears most relevant.

Under conditions of increased photosensitization in the presence of oxygen, the induction of genetic effects by 3-CP and 365-nm radiation was still less effective than that induced by 8-MOP plus the same amount of radiation (*16*).

Induction of Mitotic Chromosome Nondisjunction in Diploid Yeast

Many severe genetic defects and neoplastic changes in humans are associated with the induction of aneuploidy caused by mitotic nondisjunction (*18, 46*). Therefore, we tested the capacity of furocoumarins to induce mitotic nondisjunction (aneuploidy) in yeast. Aneuploidy, i.e., a condition in which the chromosome numbers in cells are not multiples of the haploid chromosomal set, can be observed in daughter cells generated by nondisjunction occurring during mitosis of diploid cells. One cell is

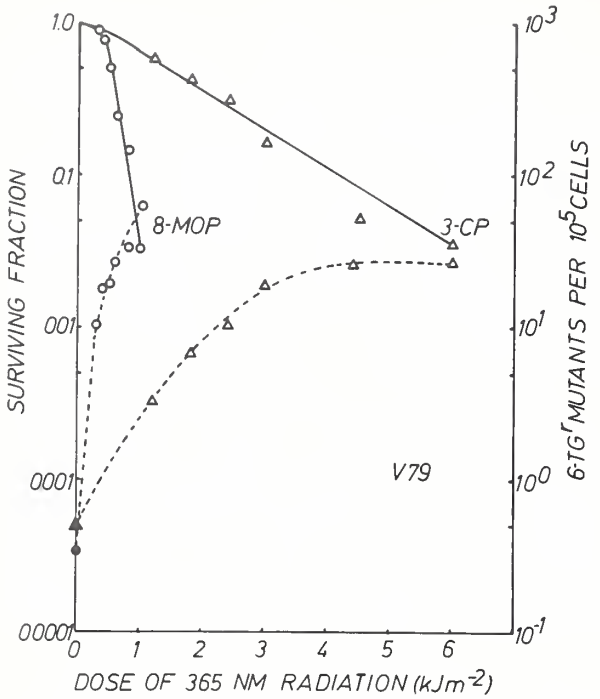

FIGURE 6.—Induction of lethal effects (*solid lines*) and of 6-TG^r cells (*dotted lines*) as a function of 365 nm radiation obtained for exponentially growing V79 Chinese hamster cells after treatment with equimolar amounts of 8-MOP (*open circles*) or 3-CP (*open triangles*) and maximum expression times for the determination of 6-TG mutants. Corresponding spontaneous frequency of 6-TG^r mutants is indicated by *closed symbols* at zero dose. Data points are average values from 2 to 6 independent experiments with maximal expression times for each dose.

monosomic, the other trisomic. Nondisjunction is not only caused by typical mutagens but also by agents interfering with the function of the spindle apparatus like colchicine or *p*-fluorophenylalanine (*17*). In view of the widespread use of furocoumarins in photochemotherapy and cosmetics, and with regard to possible side reactions of furocoumarins that may involve the spindle apparatus, we investigated the capacity of a bifunctional furocoumarin, 8-MOP, and a monofunctional furocoumarin, 4,5′-DMA, to induce mitotic nondisjunction in diploid yeast. Both furocoumarins induced monosomic cells, which indicated the induction of mitotic nondisjunction or aneuploidy (fig. 5).

As a function of 365 nm radiation (fig. 5), the bifunctional 8-MOP is clearly more effective than is the monofunctional 4,5′-DMA. Because the induction of nondisjunction is often associated with neoplastic changes (*18*) and is produced not only by agents damaging to DNA but also by those promoting tumors (*46*), the demonstration that this type of genetic alteration can be induced by psoralens is important for the evaluation of genotoxic hazards of psoralens.

Induction of Lethality and 6-Thioguanine-Resistant Mutants in V79 Chinese Hamster Cells

To determine whether psoralens exert comparable effects in higher eukaryotic systems, we performed experiments with mammalian cells in culture, using the same conditions of dosimetry of 365-nm radiation and furocoumarin concentration as with yeast cells. We used 8-MOP and 3-CP at concentrations of 50 μM to determine their lethal and genetic effects on V79 Chinese hamster cells in the presence of 365-nm radiation.

As shown in figure 6, the cells were about three to four times more resistant to photoadditions by 3-CP than by 8-MOP. The doses corresponding to 37% survival (colony-forming ability) were 0.5 and 1.8 kJ/m^2 for 8-MOP and 3-CP, respectively. The values for the lethal doses at 37% survival differ by about the same factors as the doses obtained previously in haploid yeast with the same molar concentrations of 8-MOP and 3-CP: 1.35 kJ/m^2 for 8-MOP and 3.8 kJ/m^2 for 3-CP (*15*). Moreover, these data indicate that V79 Chinese hamster cells have about twice the sensitivity that haploid yeast cells do.

The 8-MOP was clearly more effective than 3-CP for the induction of 6-TG^r forward mutants as a function of dose. For example, for the induction of 20 6-TG^r mutants/10^5 survivors in the linear part of the induction curve, a six to seven times higher dose of 3-CP is necessary than with 8-MOP. In haploid yeast, the induction of forward mutations, i.e., 50 can^r mutants/10^6 survivors differed by about the same factor of the doses needed (*15*).

Figure 7 demonstrates that, as a function of survival, the efficiency of 8-MOP to induce 6-TG^r cells is much greater than that of 3-CP. At the 37% survival level, the induced

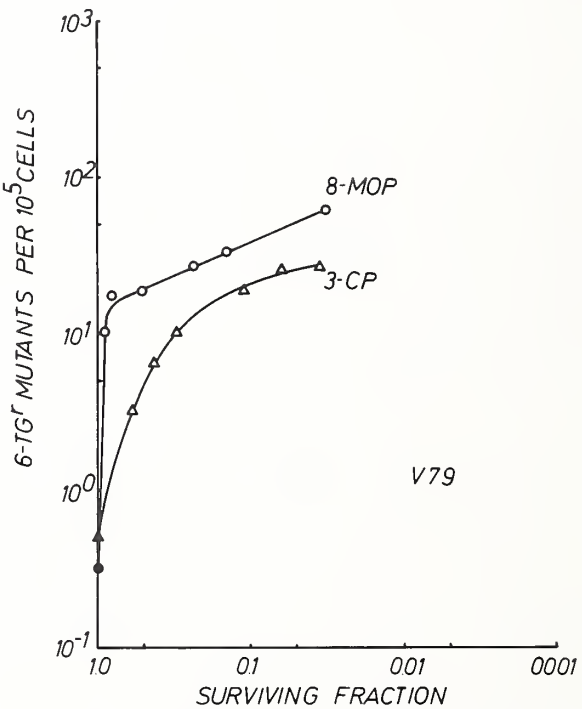

FIGURE 7.—Induction of 6-TG^r cells in exponentially growing V79 Chinese hamster cells after treatment with equimolar amounts (50 μM) of 8-MOP (*open circles*) or 3-CP (*open triangles*) as a function of survival. Frequency of 6-TG^r mutants was determined with maximal expression times used for each dose. Each *data point* represents average values from 2 to 6 independent experiments.

mutation frequencies by 8-MOP and 3-CP differ by approximately a factor of 4, which is again similar to what was reported in haploid yeast (*15*) for the induction of can^r forward mutations. These results demonstrated that the two types of furocoumarins used induced similar lethal and mutagenic effects in V79 Chinese hamster cells and in yeast.

DISCUSSION

The analysis of the mutagenic activity of psoralens plus 365-nm radiation in yeast (*S. cerevisiae*) reveals that photoreactive bifunctional furocoumarins that induce monoadditions and interstrand cross-links in DNA can be distinguished from monofunctional furocoumarins which induce only monoadditions in DNA based on their activity for inducing genetic changes. Although in such a comparison the different types of photoinduced lesions cannot be assessed in a clean separated fashion but only by indirect deduction, a certain general pattern is apparent. When the photobinding capacity of furocoumarins is known not only toward DNA in vitro but also toward DNA in living cells, e.g., 8-MOP and 3-CP in haploid yeast (*26, 47*), the relative contribution of lesions induced by monofunctional and bifunctional furocoumarins to the genetic effect induced can be established with some confidence. For instance, the survival data obtained with 8-MOP and 3-CP in haploid yeast clearly indicate that, despite the higher photobinding activity of 3-CP to cellular DNA, 8-MOP is more effective than is 3-CP in cell killing and the induction of mutations. Knowing that the photoaddition by 8-MOP consists of at least 2 types of lesions, the monoadditions and biadditions, it is reasonable for one to assume that the increased cell killing and mutagenic effect observed with 8-MOP is due to the induction of DNA interstrand cross-links. This consideration is also important for the interpretation of results obtained on the induction of cytoplasmic petite mutants in yeast (*8, 15*).

With regard to induction of nuclear reverse mutations, monofunctional furocoumarins, such as 3-CP and 4,5′-DMA, but also other monofunctional compounds, such as 3-cyanopsoralen (*7*), 3-carbomethoxypsoralen (*7*), angelicin (*38, 42*), and 5,7-dimethoxycoumarin (*42*) have been less effective than have bifunctional furocoumarins. This appears true for haploid and diploid yeast cells (*16*) with respect to the comparison of psoralen and 8-MOP with 3-CP and angelicin.

Analysis of the kinetics of the induction of reversions and the greater efficiency of bifunctional furocoumarins (per viable cell) compared with monofunctional furocoumarins indicate that the bifunctional type induces lesions that may involve development of error-prone repair. Recent results obtained with algae (*Chlamydomonas reinhardii*) on the mutagenic effects of 8-MOP and the 5′methylangelicin support this view (*48*).

Our results concerning the induction of forward mutations in eukaryotic cells (yeast and V79 Chinese hamster cells) demonstrate that certain monofunctional compounds such as 3-CP are less mutagenic than are the typical bifunctional compounds like 8-MOP. Recall here that the hypothesis of the higher mutagenicity of lesions induced by bifunctional furocoumarins compared with those induced by monofunctional furocoumarins was supported by our experiments in which we used 8-MOP and compared the effects on the induction of genetic events at high and low dose rates of 365 nm radiation (*2, 8*). With low dose rates of 8-MOP, photoinduced cross-links apparently were subject to repair processes (*2, 8*) and it was clearly less mutagenic than with high dose rates (*2, 7, 8*).

In addition, in diploid yeast, psoralen and 8-MOP were always more active than 3-CP and angelicin (*16*) with regard to the induction of mitotic crossing-over. Furthermore, because the *pso*-2 mutant of yeast, which is defective in cross-link repair (*26*), is practically immutable (*39*), we believe that the repair of cross-links is prone to error.

These results on the mutagenicity of monofunctional and bifunctional psoralens appear related to those concerning their carcinogenicity. Both 8- and 5-MOP proved to be carcinogenic in mice (*6, 49, 50*), whereas angelicin was slightly carcinogenic (Zajdela F: Personal communication), and 3-CP was noncarcinogenic (*49*) in mice. Thus our mutation data appear to agree with the idea that the presence of furocoumarin-induced cross-links confers increased lethality accompanied by increased mutagenicity (as well as the induction of mitotic crossing-over) that is likely related to the carcinogenic effects observed.

Recent biochemical studies on the inhibition of normal repair synthesis after treatment with 8-MOP and angelicin on the repair of furocoumarin-induced lesions in DNA in mammalian cells (*27–29*) showed that the photoaddition of 8-MOP or angelicin inhibit normal DNA replication (*27, 29*). Despite the presence of cross-links in 8-MOP plus UVA-treated human fibroblasts, the time course of repair replication (indicating a certain step during excision repair) was the same as that for angelicin or 254-nm UV-treated cells (*27, 28*). Excision-deficient xeroderma pigmentosum fibroblasts group A gave no response in repair replication after photoaddition of 8-MOP or angelicin, and it was concluded that the repair of monoadducts induced by these compounds shares a step with the excision scheme for pyrimidine dimers (*27*).

Based on these results, it has been argued that 8-MOP- or angelicin-induced monoadditions, like the 254-nm UV-induced pyrimidine dimers, should be both mutagenic and potentially carcinogenic in man (*28, 29*). Moreover, Hanawalt et al. (*28, 29*) hypothesized that the use of a split-dose regimen in psoralen plus longwave radiation therapy would be useful to maximize the induction of cross-links at the expense of monoadducts and to minimize the risk of cancer induction. Application of a split-dose treatment and 8-MOP conversion of some monoadducts to cross-links in skin fibroblasts (*51*) resulted in a more effective block of normal DNA replication than treatment without the split-dose regimen. When repair-deficient, excision-less (*uvr*$^-$B) mutants of *E. coli* were used, the investigators (*52*) concluded that 8-MOP-induced monoadditions were causing mutagenesis, whereas 8-MOP-induced cross-links were the major cause of cell lethality.

Using a similar re-irradiation (split-dose) protocol with 8-MOP, Bridges et al. (*53*) apparently confirmed this finding in another repair-deficient, excision-less strain of *E. coli* (WP2 *uvr*A). From this, Hanawalt et al. (*28*)

inferred that interstrand cross-links by their nature must be less readily repairable and are probably no more mutagenic than are the monoadditions (*28*). However, Bridges et al. (*53*) demonstrated that in excision-proficient, wild-type *E. coli* cells, in which cross-link repair occurs (*25*) but more likely leads to mutation than the repair of monoadditions, the induced mutation frequency tends to rise (though not greatly) when adducts are converted to cross-links. They (*53*) concluded that "cross-links appear to be rather more likely to lead to inactivation as well as to mutation induction than are the adducts from which they derive."

This theory is supported by re-irradiation experiments performed with 8-MOP and 365-nm or near UV light on the induction of methionine mutations in *Aspergillus nidulans* (*54*) and the induction of 6-TG^r mutants in V79 Chinese hamster cells (*55*). In addition, results on the mutagenic effect of furocoumarin-induced monoadducts and cross-links on bacteriophage lambda in re-irradiation experiments with 8-MOP plus light (*56*) showed that the mutagenic action of monoadducts can be detected in the absence of cross-links in DNA (after treatment with angelicin and radiation). However, Weigle mutagenesis of phage lambda produced by treatment with 8-MOP plus light depends to a large extent on photoinduced DNA cross-links.

Conclusions drawn from these data and from ours on the mutagenic effects of psoralen in yeast and in V79 Chinese hamster cells appear difficult to reconcile with the hypothesis of Hanawalt et al. (*28, 29*). Overwhelming evidence exists that the presence of furocoumarin-induced DNA cross-links results in increased mutagenicity. This may well be related to the ability of certain bifunctional furocoumarins to induce carcinogenic effects (*6*) and cell transformations in vitro (*57*). Monoadducts induced by furocoumarins also proved to be mutagenic to a lesser extent than are the furocoumarin-induced cross-links. This is likely related to the reduction (angelicin) or absence (3-CP) of carcinogenic capacity seen with certain monofunctional furocoumarins.

The proneness to error of DNA cross-links alone is difficult for one to assess because in experiments with bifunctional furocoumarins the effects of DNA cross-links are always measured concomitantly with those of the induced monoadducts. That the presence of DNA cross-links may lead to partial interference with otherwise largely error-free repair of monoadducts by decreasing its fidelity cannot be excluded totally; however, the final results as mutagenic or carcinogenic effects would remain the same.

REFERENCES

(*1*) Scott BR, Pathak MA, Mohn GR: Molecular and genetic basis of furocoumarin reactions. Mutat Res 39:29–74, 1976

(*2*) Averbeck D: Mutagenic effects of psoralens. *In* Proceedings of the Symposium on Molecular Basis of Dermatological Diseases (Pathak MA, ed). New York, London: Plenum Press. In press

(*3*) Pathak MA, Parrish JA, Fitzpatrick TB: Psoralens in photochemotherapy of skin diseases. Farmaco [Sci] 26:479–491, 1981

(*4*) Bridges B, Strauss G: Possible hazards of photochemotherapy for psoriasis. Nature 283:523–524, 1980

(*5*) Stern RS, Thibodeau LA, Kleinerman RA, et al: Risk of cutaneous carcinoma in patients treated with oral methoxsalen photochemotherapy for psoriasis. N Engl J Med 300:809–813, 1979

(*6*) Grekin DA, Epstein JH: Psoralens, UVA (PUVA) and photocarcinogenesis. Photochem Photobiol 33:957–960, 1981

(*7*) Averbeck D: Photobiology of furocoumarins. *In* Trends in Photobiology (Hélène C, Charlier M, Montenay-Garestier TH, et al, eds). New York, London: Plenum Press, 1982, pp 295–308

(*8*) Averbeck D, Magaña-Schwencke N, Moustacchi E: Genetic effects and repair in yeast of DNA lesions induced by 3-carbethoxypsoralen and other photoreactive furocoumarins of therapeutic interest. *In* Psoralens in Cosmetics and Dermatology (Cahn G, Forlot BP, Grupper C, et al, eds). New York, London: Pergamon Press, 1981, pp 143–153

(*9*) Musajo L, Rodighiero G, Caporale G, et al: Photoreactions between skin-photosensitizing furocoumarins and nucleic acids. *In* Sunlight and Man: Normal and Abnormal Photobiological Responses (Fitzpatrick TB, Pathak MA, Harber LC, et al, eds). Tokyo: Univ Tokyo Press, 1974, pp 369–387

(*10*) Dall'Acqua F: New chemical aspects of the photoreaction between psoralen and DNA. *In* Research in Photobiology (Castellani A, ed). New York, London: Plenum Press, 1977, pp 245–255

(*11*) Hearst JE: Psoralen photochemistry and nucleic acid structure. J Invest Dermatol 77:39–44, 1981

(*12*) Dall'Acqua F, Vedaldi D, Baccichetti F, et al: Photochemotherapy of skin-diseases: Comparative studies on the photochemical and photobiological properties of various mono- and bifunctional agents. Farmaco [Sci] 36:519–535, 1981

(*13*) Moustacchi E, Chanet R, Heude M: Ionizing and ultraviolet radiations: Genetic effects and repair in yeast. *In* Research in Photobiology (Castellani A, ed). New York, London: Plenum Press, 1977, pp 197–206

(*14*) Moustacchi E: Cytoplasmic and nuclear genetic events induced by UV light in strains of *Saccharomyces cerevisiae* with different UV sensitivities. Mutat Res 7:171–185, 1969

(*15*) Averbeck D, Averbeck S, Dall'Acqua F: Mutagenic activity of three monofunctional and three bifunctional furocoumarins in yeast (*Saccharomyces cerevisiae*). Farmaco [Sci] 36:492–505, 1981

(*16*) Averbeck D, Moustacchi E: Genetic effect of 3-carbethoxypsoralen, angelicin, psoralen and 8-methoxypsoralen plus 365-nm irradiation in *Saccharomyces cerevisiae.* Induction of reversions, mitotic crossing-over, gene conversion and cytoplasmic "petite" mutations. Mutat Res 68:133–148, 1979

(*17*) Parry JM, Zimmermann FK: Detection of monosomic colonies produced by mitotic nondisjunction in the yeast *Saccharomyces cerevisiae.* Mutat Res 36:49–66, 1976

(*18*) Parry JM, Sharp D, Parry EM: Detection of mitotic and meiotic aneuploidy in the yeast *Saccharomyces cerevisiae.* Environ Health Perspect 31:97–111, 1979

(*19*) Zimmermann FK, von Borstel RC, von Halle ES, et al: Testing of chemicals for genetic activity with *Saccharomyces cerevisiae:* A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutat Res 133:199–244, 1984

(*20*) Ogur H, John S, Nagai S: Tetrazolium overlay technic for population studies of respiration deficiency in yeast. Science 125:928–929, 1957

(*21*) QUEVAL P, BISAGNI E: New synthesis of psoralen related compounds. Eur J Med Chem 9:335–340, 1974

(*22*) AVERBECK D, BISAGNI E, MARQUET JP, et al: Photobiological activity in yeast of derivatives of psoralen substituted at the 3,4-and/or the 4′,5′-reaction site. Photochem Photobiol 30:547–555, 1979

(*23*) ARLETT CF, HARCOURT SA: Expression time and spontaneous mutability in the estimation of induced mutation frequency following treatment of Chinese hamster cells by UV light. Mutat Res 16:301–306, 1972

(*24*) SONG P-S, TAPLEY KJ JR: Photochemistry and photobiology of psoralens. Photochem Photobiol 29:11–97, 1979

(*25*) COLE RS, SINDEN RR, YOAKUM GH, et al: On the mechanism for repair of cross-linked DNA in *E. coli* treated with psoralen and light. *In* DNA Repair Mechanisms (Hanawalt PC, Friedberg EC, Fox CF, eds). New York: Academic Press, 1978, pp 287–290

(*26*) MAGAÑA-SCHWENCKE N, HENRIQUES JA, CHANET R, et al: The fate of 8-methoxypsoralen photoinduced crosslinks in nuclear and mitochondrial yeast DNA: Comparison of wild-type and repair-deficient strains. Proc Natl Acad Sci USA 79:1722–1726, 1982

(*27*) KAYE J, SMITH CA, HANAWALT PC: DNA repair in human cells containing photoadducts of 8-methoxypsoralen or angelicin. Cancer Res 40:696–702, 1980

(*28*) HANAWALT PC, KAYE J, SMITH CA, et al: Cellular responses to psoralen adducts in DNA. *In* Psoralens in Cosmetics and Dermatology (Cahn G, Forlot BP, Grupper C, et al, eds). New York: Pergamon Press, 1981, pp 133–142

(*29*) HANAWALT PC, LIU SC, PARSONS CS: DNA repair responses in human skin cells. J Invest Dermatol 77:86–90, 1981

(*30*) AVERBECK D, MOUSTACCHI E: 8-Methoxypsoralen plus 365 nm light effects and repair in yeast. Biochim Biophys Acta 395:393–404, 1975

(*31*) AVERBECK D, MOUSTACCHI E, BISAGNI E: Biological effects and repair of damage photoinduced by a derivative of psoralen substituted at the 3,4 reaction site. Photoreactivity of this compound and lethal effect in yeast. Biochim Biophys Acta 518:464–481, 1978

(*32*) HENRIQUES JA, MOUSTACCHI E: Isolation and characterization of *pso* mutants sensitive to photo-addition of psoralen derivatives in *Saccharomyces cerevisiae*. Genetics 95:273–288, 1980

(*33*) ———: Sensitivity to photo-addition of mono- and bifunctional furocoumarins of X-ray sensitive mutants of *Saccharomyces cerevisiae*. Photochem Photobiol 31:555–562, 1980

(*34*) ———: Interactions between mutations for sensitivity to psoralen photoaddition (*pso*) and to radiation (*rad*) in *Saccharomyces cerevisiae*. J Bacteriol 148:248–256, 1981

(*35*) AVERBECK D, CHANDRA P, BISWAS RK: Structural specificity in the lethal and mutagenic activity of furocoumarins in yeast cells. Radiat Environ Biophys 12:241–252, 1975

(*36*) HENRIQUES JA, CHANET R, AVERBECK D, et al: Lethality and "petite" mutation induced by the photoaddition of 8-methoxypsoralen in yeast. MGG 158:63–73, 1977

(*37*) SWANBECK G, THYRESSON M: Induction of respiratory-deficient mutants in yeast by psoralen and light. J Invest Dermatol 63:242–244, 1974

(*38*) GRANT EL, VON BORSTEL RC, ASHWOOD-SMITH MJ: Mutagenicity of cross-links and monoadducts of furocoumarins (psoralen and angelicin) induced by 360-nm radiation in excision-repair-defective and radiation-insensitive strains of *Saccharomyces cerevisiae*. Environ Mutagen 1:55–63, 1979

(*39*) CASSIER C, CHANET R, HENRIQUES JA, et al: The effects of three *pso* genes on induced mutagenesis: A novel class of mutationally defective yeast. Genetics 96:841–857, 1980

(*40*) ECKARDT F, HAYNES RH: Kinetics of mutation induction by ultraviolet light in excision-deficient yeast. Genetics 85:225–247, 1977

(*41*) KUNZ BA, ECKARDT F, HAYNES RH: Manifestation of inducible components in dose-response curves for mutation and recombination. Presented at the Symposium on Molecular and Cellular Mechanism of Mutagenesis, Gatlinburg, Tenn., April 6–9, 1981

(*42*) AVERBECK D, MOUSTACCHI E: Decreased photo-induced mutagenicity of mono-functional as opposed to bi-functional furocoumarins in yeast. Photochem Photobiol 31:475–478, 1980

(*43*) DUBERTRET L, AVERBECK D, BENSASSON R, et al: Photophysical, photochemical, photobiological and phototherapeutic properties of 3-carbethoxypsoralen. *In* Psoralens in Cosmetics and Dermatology (Cahn G, Forlot BP, Grupper C, et al, eds). New York: Pergamon Press, 1981; pp 245–256

(*44*) RONFARD-HARET JC, AVERBECK D, BENSASSON RV, et al: Some properties of the triplet excited state of the photosensitizing furocoumarin, 3-carbethoxypsoralen. Photochem Photobiol 35:479–489, 1982

(*45*) SPIKES JD: Round table summary: Endogenous, exogenous inhibitors and sensitizers. Fundamental aspects. *In* Trends in Photobiology (Hélène C, Charlier M, Montenay-Garestier TH, et al, eds). New York, London: Plenum Press, 1981, pp 109–121

(*46*) PARRY JM, PARRY EM, BARRET JC: Tumour promoters induce mitotic aneuploidy in yeast. Nature 294:263–265, 1981

(*47*) MAGAÑA-SCHWENCKE N, AVERBECK D, HENRIQUES JA, et al: Absence de pontages interchaines dans l'ADN traité par le 3-carbéthoxypsoralène et une irradiation à 365 nm. C R Acad Sci (Paris) 291:207–210, 1980

(*48*) ABEL G, SCHIMMER O: Mutagenicity and toxicity of furocoumarins: Comparative investigations in 2 test systems. Mutat Res 90:451–461, 1981

(*49*) DUBERTRET L, AVERBECK D, ZAJDELA F, et al: Photochemotherapy (PUVA) of psoriasis using 3-carbethoxypsoralen, a compound noncarcinogenic in mice. Br J Dermatol 101:379–389, 1979

(*50*) ZAJDELA F, BISAGNI E: 5-Methoxypsoralen, the melanogenic additive in sun-tan preparations is tumorigenic in mice exposed to 365 nm UV radiation. Carcinogenesis 2:121–127, 1981

(*51*) POHL J, CHRISTOPHERS E: Photoinactivation of skin fibroblasts by fractionated treatment with 8-methoxypsoralen and UVA. J Invest Dermatol 73:176–179, 1979

(*52*) SEKI T, NOZU K, KONDO S: Differential causes of mutation and killing in *Escherichia coli* after psoralen plus light treatment: Monoadducts and cross-links. Photochem Photobiol 27:19–24, 1978

(*53*) BRIDGES BA, MOTTERSHEAD RP, KNOWLES A: Mutation induction and killing of *Escherichia coli* by DNA adducts and crosslinks: A photobiological study with 8-methoxypsoralen. Chem Biol Interact 27:221–233, 1979

(*54*) SCOTT BR, MALEY MA: Mutagenicity of monoadducts and cross-links induced in *Aspergillus nidulans* by 8-methoxypsoralen plus 365 nm radiation. Photochem Photobiol 34:63–67, 1981

(*55*) BABUDRI N, PANI B, VENTURINI S, et al: Mutation induction

and killing of V79 Chinese hamster cells by 8-methoxypsoralen plus near-ultraviolet light: Relative effects of monoadducts and cross-links. Mutat Res 91:391–394, 1981

(*56*) BELOGUROV AA, ZAVILGELSKI GB: Mutagenic effect of furocoumarin monoadducts and cross-links on bacteriophage lambda. Mutat Res 84:11–15, 1981

(*57*) BURGER PP, SIMONS JW: Mutagenicity and carcinogenicity of 8-MOP/UVA in cell cultures. Bull Cancer (Paris) 65:281–282, 1978

Repair of Furocoumarin Adducts in Mammalian Cells[1, 2]

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ABSTRACT—We studied DNA repair in cultured mammalian cells treated with the furocoumarins 8-methoxypsoralen (8-MOP), aminomethyl trioxsalen, or angelicin and irradiated with near UV light. The amount of DNA cross-linked by 8-MOP in normal human cells decreased by about one-half in 24 hours after treatment; no decrease was observed in xeroderma pigmentosum cells, group A. At present, it is not known to what extent this decrease represents complete repair events at the sites of cross-links. Furocoumarin adducts elicited excision repair in normal human and monkey cells but not in xeroderma pigmentosum group A cells. This excision repair resembled in several aspects that elicited by pyrimidine dimers, formed in DNA by irradiation with 254-nm UV light; however, it appeared that for at least 8-MOP and aminomethyl trioxsalen, removal of adducts was not as efficient as was the removal of pyrimidine dimers. We also compared repair in the 172-base-pair repetitive α-DNA component of monkey cells to repair in the bulk of the genome. Although repair elicited by pyrimidine dimers in α-DNA was the same as in the bulk DNA, that following treatment of cells with either aminomethyl trioxsalen or angelicin and near UV was markedly deficient in α-DNA. This deficiency reflected the removal of fewer adducts from α-DNA after the same initial adduct frequencies. These results could mean that each furocoumarin may produce several structurally distinct adducts to DNA in cells and that the capacity of cellular repair systems to remove these various adducts may vary greatly. Alternately, the chromatin structure of α-DNA may restrict the access of enzymes that recognize or repair, or both, furocoumarin adducts in mammalian cells. — Natl Cancer Inst Monogr 66: 137–142, 1984.

Furocoumarins intercalate into the DNA helix and, upon subsequent photoactivation by UVA, may form adducts to pyrimidines, principally at the 5- and 6-carbons of thymine. These are the same positions involved in the cyclobutyl pyrimidine dimer formed in DNA upon absorption of UVC. Certain of the monoadducts of furocoumarins with a linear arrangement of their ring system (psoralens) may react with an appropriately positioned thymine in the opposite DNA strand, thus forming an interstrand cross-link. Some furocoumarins, such as angelicin, form only monoadducts because the angular arrangement of the rings precludes proper juxtaposition of the unreacted end of the monoadduct (*1*).

These covalent adducts pose blocks to normal DNA functions and elicit the cellular recovery response termed "excision repair," in which a short stretch of a DNA strand containing damage is excised and replaced by means of DNA synthesis with the use of the undamaged complementary strand as template. The operations of this system in the repair of DNA containing pyrimidine dimers have been extensively studied (*2*). Conceptually, it would appear that the furocoumarin monoadduct, like various other monofunctional chemical adducts, might be repaired by the same enzymatic system used for pyrimidine dimers. However, repair of cross-links obviously poses additional problems because damage is present in opposite DNA strands at sites only 1 nucleotide apart.

Inasmuch as unrepaired or improperly repaired DNA damage is thought to be implicated in cell death, mutagenesis, and carcinogenesis (*2*), details of the processes involved in repair of the various furocoumarin adducts are important in our understanding of the means by which these agents are effective in treatment of various dermatologic conditions.

MATERIALS AND METHODS

The details of all the materials and methods utilized in this work were described in (*3–5*) and references therein.

RESULTS

Removal of Cross-links

Little is known about the process that removes psoralen cross-links from the DNA of mammalian cells. To estimate the frequency of cross-links, we used an assay based on the rapid renaturation of molecules containing cross-links and their subsequent resistance to the single-strand specific nuclease, S_1. To evaluate removal of cross-links without the need for evaluation of the molecular weights of the DNA analyzed, we harvested and froze cells that were labeled in DNA with different isotopes either immediately after treatment with 8-MOP and UVA or 24 hours later. They were then combined before preparation and

ABBREVIATIONS: UVA = long wavelength UV radiation with a maximal output at 360 nm; UVC = 254-nm UV radiation; 8-MOP = 8-methoxypsoralen; AMT = 4′-aminomethyl-4,5′,8-trimethylpsoralen hydrochloride; BrdUrd = 5′-bromodeoxyuridine; FdUrd = fluorodeoxyuridine; kJ = kilojoule(s).

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analysis of the DNA. We found that in normal human fibroblasts the fraction of DNA in the cross-linked form diminished by about one-half in 24 hours; the initial cross-link frequency in this experiment was about 3–5 per 10^8 daltons DNA. We want to emphasize that this assay measures only the loss of cross-linking of the DNA and does not demonstrate restitution of DNA integrity. For example, introduction of only a single nick at or near each cross-link would result in a decline in cross-linking but obviously would not in itself restore biologic function. However, this process does share at least one step with monoadduct repair; removal of cross-linking was absent in xeroderma pigmentosum cells (complementation group A) but present in xeroderma pigmentosum variant cells. This finding correlates with the repair characteristics of these cells with respect to removal of pyrimidine dimers (*3*).

Repair Replication in Cells Containing Furocoumarin Adducts

The repair replication elicited by 8-MOP or angelicin and UVA in normal human cells was absent in xeroderma pigmentosum cells of complementation group A (*3*). This absence demonstrates that the pathway for repair of monofunctional furocoumarin adducts shares at least one step with that for pyrimidine dimers. We attempted to compare in detail several features of repair replication elicited by various furocoumarin adducts with those elicited by pyrimidine dimers.

The size of the repair patches synthesized in response to UVC, 8-MOP, AMT, and angelicin is the same (*3, 4*). This similarity allows us to relate repair synthesis measurements to numbers of repair events completed and, presumably, to the removal of monoadduct damage. The time course of excision repair is also similar for repair of all these adducts (*3, 4*). Measurements of the initial rate of repair in cells containing saturating levels of pyrimidine dimers with or without the additional presence of AMT adducts showed that the maximum initial rate observed with pyrimidine dimers was not increased by the presence of the furocoumarin adducts (*5*). Thus the rate limiting step may be the same for repair of these different lesions.

These results suggest that the repair pathway for removal of furocoumarin monoadducts is similar or identical to that used for pyrimidine dimers. However, repair of DNA containing these lesions is not identical in all respects. The UVA dose responses for the initial rate of repair replication for AMT, 8-MOP, and angelicin (fig. 1) show clear differences. The high solubility and binding affinity of AMT for DNA allows the introduction of a high frequency of adducts at low UVA doses. The initial rate of repair observed in cells treated with AMT and UVA reaches a plateau at about 35% of the maximum value obtainable in cells irradiated with UVC. This plateau does not represent saturation of the DNA with adducts. We estimated adduct frequencies by measuring the cross-link frequency in purified DNA both before and after irradiation with saturating amounts of UVA and by assuming that about one-half the monoadducts present can be converted in this way to cross-links. These measurements have shown that, in cells treated with high AMT concentrations, the maximum rate of repair is attained at high monoadduct-to-cross-link ratios (>80:1) and low absolute cross-link frequencies. Over the UVA dose range shown in the figure, total adducts increased nearly linearly, and the monoadduct-to-cross-link ratio remained high. When compared with frequencies of pyrimidine dimers, it is clear that, under these conditions, the AMT monoadducts are not removed as efficiently; in each instance, the initial repair rate reached one-half its maximum value at about the same adduct frequency, i.e., 30–35/10^8 daltons DNA, but the actual rate of repair elicited by AMT adducts was only about 30% of that elicited by pyrimidine dimers. Because the time course for repair is the same, only about 30% as many AMT monoadducts as pyrimidine dimers will have been removed at any given time after damage.

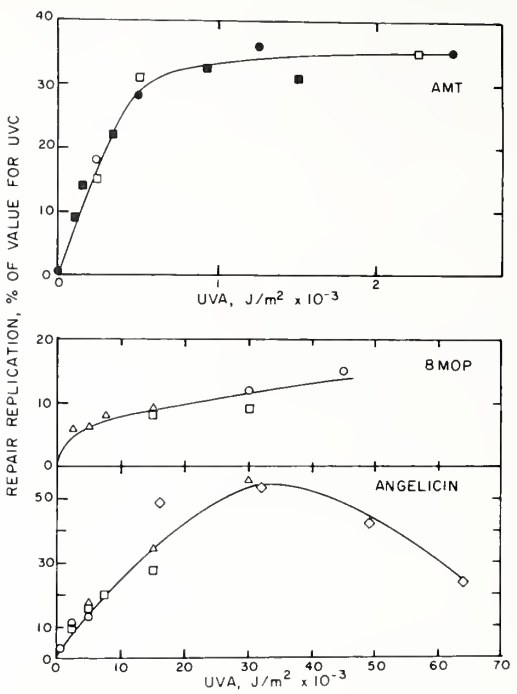

FIGURE 1.—Dose response for repair replication in T98G cells exposed to UVA in the presence of 200 or 400 μg AMT/ml, 25 μg 8-MOP/ml, or 25 μg angelicin/ml and incubated for 4 hr. Repair is expressed as the percent of the value obtained for cells irradiated with 50 J UVC/m^2 in the same experiment. Different symbols represent different experiments. Figure is reproduced with permission of the publisher (*5*).

With 8-MOP and angelicin, poor solubility and low binding affinity combine to make introduction of a high frequency of adducts dependent on delivery of large doses of UVA. We were able to do this by irradiating cells at 4° C; control experiments showed that holding cells at this low temperature did not affect repair in cells irradiated with UVC.

With 8-MOP, even at high UVA fluences, the repair rate remained low in comparison to that elicited by UVC (fig. 1). Under these conditions, lower monoadduct-to-cross-link ratios are obtained (10:1 to 2:1). Preliminary data suggest that the low repair levels are due both to our inability to produce comparable frequencies of monoadducts and to less efficient removal of the monoadducts.

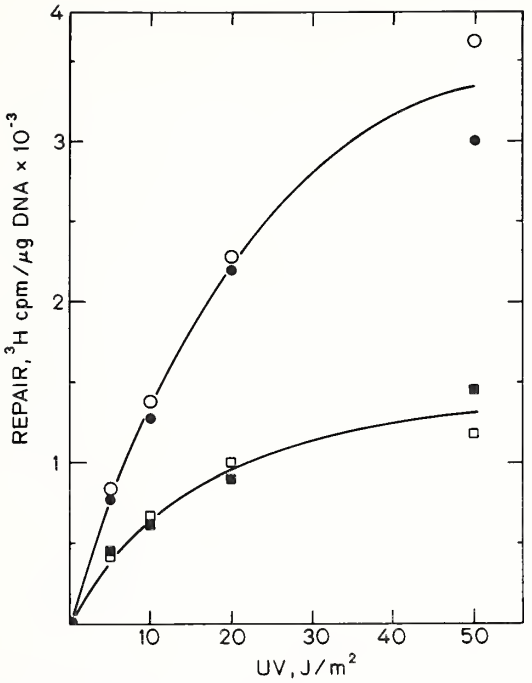

FIGURE 2.—Repair synthesis in α- and bulk DNA in UVC-irradiated BS-C-1 cells. Cells were irradiated with UVC and incubated for 4 hr (*squares*) or 24 hr (*circles*) in medium containing 30 μCi [^{3}H]dThd/ml, 50 μ*M* BrdUrd, and 5 μ*M* FdUrd. The amount of repair synthesis (tritium incorporated/microgram DNA) was measured in α-DNA (*solid symbols*) and bulk DNA (*open symbols*) as described in (*4*). Figure is reproduced with permission of the publisher (*4*). cpm = counts/minute.

The dose response for initial repair rate in cells treated with low AMT concentrations resembled that for cells treated with 8-MOP.

With angelicin, higher values for repair rates were obtained, but the repair rate declined at doses of UVA greater than 30 kJ/m^2. At doses greater than this, the UVA itself inhibits considerably the repair of UVC damage, and cells rapidly lose viability. It is likely that, under these conditions, additional phototoxicity of furocoumarins not related to DNA damage may also inhibit the repair process. Extrapolation of the curve at lower doses suggests that angelicin damage might be repaired as efficiently as are pyrimidine dimers. We obviously cannot calculate adduct frequencies by cross-link measurements in this case; preliminary measurements with the use of a technique for separating adducts from bases in enzymatic digests of DNA suggest that angelicin monoadducts are repaired more efficiently than are those of AMT or 8-MOP.

These results may lead one to think that the presence of cross-links greatly reduces the cell's ability to remove monoadducts; however, this does not appear to be true. We examined the effect of washing 8-MOP or AMT out of the cells after the UVA irradiation and then re-irradiating the cells to increase the cross-link frequency. The initial rate of repair was only slightly diminished by such treatment. Also, the rate of repair in cells treated with AMT reached a plateau rather than declining with increasing UVA (fig. 1). Over the range of UVA shown in this figure, the cross-link frequency increased by a factor of 20 at the highest AMT concentration used. Finally, repair in cells irradiated with saturating levels of UVC and then treated with AMT at a high concentration followed by a UVA irradiation designed to maximize cross-links only diminished the repair rate slightly.

Repair in the α-DNA Sequence of Monkey Cells

We have begun to characterize damage and repair in a specific DNA sequence to characterize more fully the biologic consequences of various furocoumarin adducts. Primate genomes contain a repeated DNA sequence termed "α," which, in African green monkey cells, seems particularly attractive for repair studies. In these cells, α comprises about 15% of the genome (*6–8*). This DNA has a base composition similar to that of the bulk of the DNA (*6*) but is apparently not transcribed (*9*). The basic repeating unit of α in these cells is a sequence of 172 base pairs arranged primarily in tandem arrays on several chromosomes (*7, 10*). Digestion of purified DNA with the restriction endonuclease *Hind*III and separation of the DNA fragments by electrophoresis through agarose or acrylamide gels allow the isolation of 8% of the DNA in the form of these 172-base pair α monomer fragments, which have been shown to be at least 90% homogeneous with respect to sequence (*11*).

We irradiated confluent BS-C-1 cells with various doses of UVC and determined the amount of repair replication per unit DNA in both α- and bulk DNA. We observed no significant differences in repair synthesis between α- and bulk DNA over the dose range studied (fig. 2). We also measured the frequencies of pyrimidine dimers in α- and bulk DNA and found that for both species, 0.3% of the thymine was in pyrimidine dimers after a UVC dose of 50 J/m^2. Thus both the frequency of formation and the rate and extent of repair of pyrimidine dimers were about the same in α- and bulk DNA.

We also examined repair replication in α- and bulk DNA in cells treated with AMT and UVA. Here, in contrast to

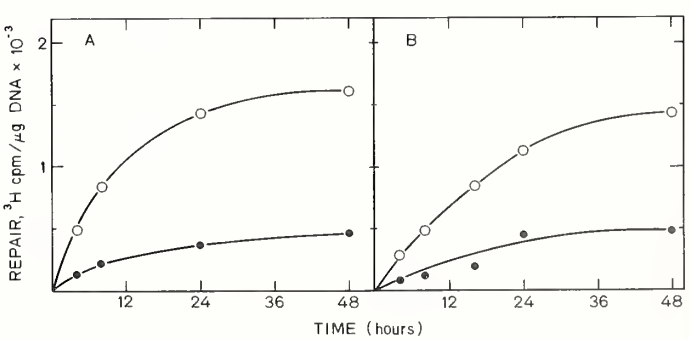

FIGURE 3.—Time course of excision repair in α- and bulk DNA after treatment of cells with AMT or angelicin. We treated BS-C-1 cells with (A) 100 μg AMT/ml and 2.5 kJ UVA/m^2 or (B) 25 μg angelicin/ml and 15 kJ UVA/m^2 and incubated them in medium containing 30 μCi [^{3}H]dThd/ml, 50 μ*M* BrdUrd, and 5 μ*M* FdUrd. The amount of repair synthesis was measured in α-DNA (*closed symbols*) and bulk DNA (*open symbols*) as described in (*4*). Figure is reproduced with permission of the publisher (*4*). cpm = counts/minute.

TABLE 1.—*Adduct frequencies and excision repair in α-DNA and bulk DNA after treatment of cells with AMT*[a]

AMT concentration, μg/ml	Ratio of: Adduct frequency in α-DNA: adduct frequency in bulk DNA	Ratio of: Repair in α-DNA: repair in bulk DNA
6.25	0.9	0.3
12.5	0.8	0.3
25	0.8	0.3

[a] Cells were exposed to 2.5 kJ UVA/m^2. Table is reproduced with permission of the publisher (*4*).

our result with UVC, the ratio of repair replication in α-DNA to that in the bulk DNA was about 0.3 over a 48-hour period (fig. 3A), the same result was observed with angelicin (fig. 3B). Thus, unlike the repair of pyrimidine dimers, the repair replication in α-DNA after furocoumarin damage was significantly lower than that in the bulk DNA.

To determine if initial adduct frequencies differed between α- and bulk DNA, we treated cells with [^{3}H]AMT, exposed them to UVA, and measured the amount of radioactivity covalently bound to either α- or bulk DNA. We also measured repair replication in α- and bulk DNA of cells treated with the same drug concentration and UVA exposure and incubated for 24 hours. The ratio of AMT bound in α-DNA to that in bulk DNA was 0.8–0.9 at each drug concentration tested (table 1). When we treated purified BS-C-1 DNA with 20 μg [^{3}H]AMT/ml and exposed it to 5 kJ UVA/m^2, the ratio of bound radioactivity in α to that in the bulk DNA was 0.8, similar to the result obtained with intact cells.

Although the adduct frequencies for α- and bulk DNA differed only slightly, the ratios of repair replication in α-DNA to that in bulk DNA in the cells treated with similar concentrations of nonradioactive drug were again only 0.3 (table 1). Thus over a wide range of conditions with 2 furocoumarins, only 30% as much repair replication was observed in α-DNA compared with that in bulk DNA, even though initial adduct frequencies were similar.

We obtained direct evidence that the lower amount of repair replication observed in α-DNA reflects the removal of fewer adducts per unit α-DNA by measuring the removal of bound [^{3}H]AMT from cellular DNA. In the experiment shown in table 1, we also prepared DNA from cells treated with the radioactive drug then incubated for 24 hours.

TABLE 2.—*Removal of AMT adducts in DNA by BS-C-1 cells*

DNA	Adducts/10^8 daltons DNA: Initial frequency	After 24 hr	No. removed
Bulk	25	18	7
α	23	21	2

$$\frac{\text{Removal, } \alpha}{\text{Removal, bulk}} = 0.3.$$

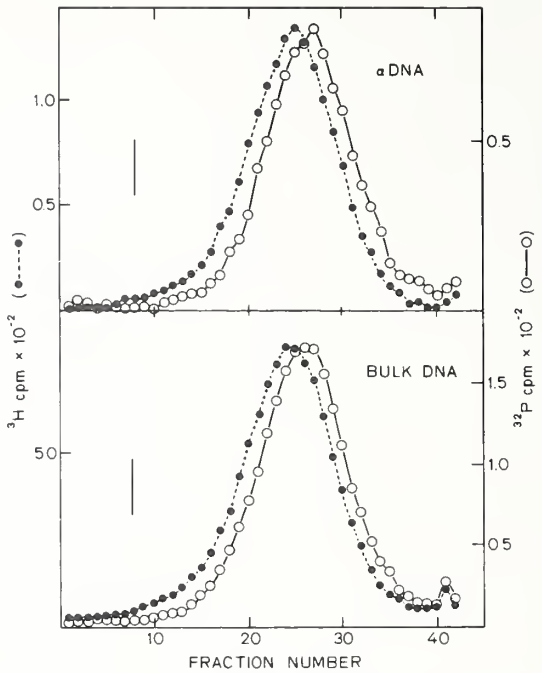

FIGURE 4.—Distribution of repair patch size in α- and bulk DNA in cells treated with AMT. The BS-C-1 cells prelabeled with phosphorus-32 were treated with 100 μg AMT/ml and 2.5 kJ UVA/m^2 and incubated for 24 hr in medium containing 30 μCi [^{3}H]dThd/ml, 100 μ*M* BrdUrd, and 10 μ*M* FdUrd. Parental density α- and bulk DNA were isolated and then centrifuged to equilibrium in alkaline cesium chloride gradients. Bulk DNA was first sonicated to a number-average molecular weight of about 160 nucleotides. *Vertical lines* indicate the position of fully BrdUrd-substituted DNA. Figure is reproduced with permission of the publisher (*4*). cpm = counts per minute.

From the specific activities of the DNA and the specific activity of the drug, we calculated the number of adducts per unit DNA in α- and bulk DNA, both initially and also after the 24-hour incubation (table 2). The number of adducts removed from α-DNA was only 0.28 as much as from bulk DNA, a value in agreement with the result obtained for repair replication, i.e., 0.3.

We measured the patch size for excision repair in both α- and bulk DNA in cells irradiated with UVC or treated with AMT and UVA and found it was about 20 nucleotides for α-DNA and bulk DNA for repair of both types of damage (the data for AMT are shown in fig. 4). This is further evidence that the lower repair replication observed in α-DNA after furocoumarin damage was due to removal of fewer adducts, not to a different mode of excision repair.

Thus not only are certain furocoumarin adducts repaired less efficiently than are pyrimidine dimers in the bulk of the DNA, but at least 1 DNA sequence found in primate cells appears to be even more refractory to repair of AMT and angelicin adducts than is the bulk of the DNA.

DISCUSSION

Our studies on the metabolism of furocoumarin adducts in cultured human and monkey cells have shown that they

inhibit semiconservative DNA synthesis and that both monofunctional and bifunctional adducts are repaired to various extents. The amount of DNA cross-linking mediated by psoralen adducts diminishes in vivo in normal human cells over a 24-hour period by a pathway sharing at least one enzymatic step with the pathway for repair of DNA containing monofunctional adducts or pyrimidine dimers. However, we do not know whether this decrease in the amount of cross-linking reflects a process that results in complete restitution of intact DNA strands. Evidence from genetic experiments with *Escherichia coli* (*12*) and yeast (*13*) has shown that cross-link repair may require both excision and recombination systems. The repair of DNA containing monoadducts shares many properties with that of DNA containing pyrimidine dimers: The patch size produced by the system, the time course of repair synthesis following damage, and the rate limiting step are apparently the same. However, the repair of certain furocoumarin adducts is not as efficient on a per adduct basis as is repair of pyrimidine dimers. Monoadducts of AMT and possibly 8-MOP are apparently repaired to a lesser extent, on average, than are pyrimidine dimers.

We have also shown that excision repair in the highly repeated α-sequence of UVC-irradiated cultured monkey cells is the same as it is in the bulk of the genome. In contrast, in cells containing furocoumarin adducts, only 30% as much repair synthesis occurs in α-DNA as in the bulk DNA. We demonstrated that this reflects the removal of fewer adducts from α-DNA after approximately the same amount of damage.

Although the amount of repair synthesis observed in α-DNA from cells treated with furocoumarins was less than in bulk DNA, the time course of repair synthesis was the same in α- and bulk DNA. Furthermore, the size of the repair patches was the same in α- as in bulk DNA. These observations indicate that the repair of furocoumarin adducts in α-DNA occurs in the same manner and with the same kinetics as in the rest of the genome, but a substantial fraction of the adducts formed in α-DNA are not repairable. Our result with angelicin, which does not form cross-links, rules out differential formation or repair of interstrand cross-links in α-DNA as an explanation for the lesser amount of repair that we observe. However, the furocoumarins can form a number of structurally distinct monoadducts with DNA (*14*). It is possible that the sequence or chromatin structure of α-DNA mediates the formation of a high proportion of nonrepairable adducts. The frequency of formation of pyrimidine dimers depends on the nucleotides surrounding each potential dimer site (*15*). It seems likely that this will also prove to be true for furocoumarins and that all furocoumarin adducts will not be equally well recognized by cellular repair enzymes. Alternately, the same spectra of furocoumarin adducts could form in α-DNA as in bulk DNA, but cellular repair enzymes may have restricted access to α-DNA.

Our studies with α-DNA will serve as a model system for understanding the details of furocoumarin repair in primate cells. Because cultured monkey cells show properties similar to those of cultured human cells, this system should be a valid model for the study of human repair processes. Analysis of why furocoumarin adducts are poorly removed from α-DNA should provide insight into the specifics of repair of DNA containing these lesions in general. The α-DNA component may reflect, in an amplified way, the nonrepairable state of many furocoumarin adducts. In addition, the fact that certain portions of the genome are especially poorly repaired after furocoumarin damage may be important in the manner by which these drugs kill cells and cause mutations.

So far, our studies of the repair of interstrand cross-links have not been extensive. In most experiments, we deliberately used compounds or experimental conditions designed to maximize the formation of monoadducts, the removal of which would appear to be mediated by systems that also act on DNA containing other adducts confined to 1 DNA strand, such as pyrimidine dimers and large nucleophilic chemical species. These studies have 1) shown that repair of even these closely related furocoumarin monoadducts shows some significant differences and 2) demonstrated the need for quantitative assessment of the formation and removal of chemically distinct adducts. To date, therapeutic treatments have usually involved low drug concentrations and high UVA doses (*16*), conditions which favor cross-link formation with the psoralens (*17*). The biologic consequences of the presence of cross-links and the details of the mechanisms by which the cell responds to them are obviously important to the optimal use of furocoumarins therapeutically. We hope that use of the α-DNA system and more sensitive assays for the various chemically distinct adducts produced by furocoumarins will aid in elucidation of the details of formation and repair of the different adducts. We hope to gain insight into the means by which different biologic effects may result from various combinations of furocoumarin adducts in DNA.

NOTE ADDED IN PROOF

Since the time of this meeting in March 1982, we have made considerable progress in our understanding of the deficient repair of α-DNA. Please see (*18, 19*).

REFERENCES

(*1*) Song P-S, Tapley KJ: Photochemistry and photobiology of psoralens. Photochem Photobiol 29:1177–1197, 1979

(*2*) Hanawalt PC, Cooper PK, Ganesan AK, et al: DNA repair in bacteria and mammalian cells. Annu Rev Biochem 48:783–836, 1979

(*3*) Kaye J, Smith CA, Hanawalt PC: DNA repair in human cells containing photoadducts of 8-methoxypsoralen or angelicin. Cancer Res 40:696–702, 1980

(*4*) Zolan ME, Cortopassi GA, Smith CA, et al: Deficient repair of chemical adducts in α-DNA of monkey cells. Cell 28:613–619, 1982

(*5*) Hanawalt PC, Kaye J, Smith CA, et al: Cellular responses to psoralen adducts in DNA. *In* Psoralens in Cosmetics and Dermatology (Cahn J, Forlot P, Grupper C, et al, eds). New York: Pergamon Press, 1981, pp 133–142

(*6*) Maio JJ: DNA strand reassociation and polyribonucleotide binding in the African green monkey, *Cercopitheius acthiops*. J Mol Biol 56:579–595, 1971

(*7*) Fittler F: Analysis of the α-satellite DNA from African

green monkey cells by restriction endonucleases. Eur J Biochem 74:343–352, 1977

(*8*) SINGER DS: Arrangement of a highly repeated DNA sequence in the genome and chromatin of the African green monkey. J Biol Chem 254:5506–5514, 1979

(*9*) KUFF EL, FERDINAND FJ, KHOURY G: Transcription of host-substituted simian virus 40 DNA in whole cells and extracts. J Virol 25:28–36, 1978

(*10*) BROWN FL, MUSICH PR, MAIO JJ: The repetitive sequence structure of component α DNA and its relationship to the nucleosomes of the African green monkey. J Mol Biol 131:777–799, 1979

(*11*) ROSENBERG H, SINGER M, ROSENBERG M: Highly reiterated sequences of simiansimiansimiansimiansimian. Science 200:394–402, 1978

(*12*) COLE RS, SINDEN RR, YOAKUM GH, et al: On the mechanism for repair of cross-linked DNA in *E. coli* treated with psoralen and light. *In* DNA Repair Mechanisms (Hanawalt PC, Friedberg EC, Fox CF, eds). New York: Academic Press, 1978, pp 287–290

(*13*) JACHYMCZYK WV, VON BORSTEL RC, MOWAT MR, et al: Repair of interstrand cross-links in DNA of *Saccharomyces cerevisiae* requires two systems for DNA repair: The RAD 3 system and the RAD 51 system. MGG 182:196–205, 1981

(*14*) STRAUB K, KANNE D, HEARST JE, et al: Isolation and characterization of pyrimidine-psoralen photoadducts from DNA. J Am Chem Soc 103:2347–2355, 1981

(*15*) HASELTINE WA, GORDON LK, LINDAN CP, et al: Cleavage of pyrimidine dimers in specific DNA sequences by a pyrimidine dimer DNA-glycosylase of *M. luteus*. Nature 285:634–641, 1980

(*16*) ROENIGK HH JR: The U.S. PUVA-48 center cooperative study. *In* Psoralens in Cosmetics and Dermatology (Cahn J, Forlot P, Grupper C, et al, eds). New York: Pergamon Press, 1981, pp 211–222.

(*17*) ISAACS S, HEARST JE: In vitro characterization of the reaction of four psoralen derivatives with DNA. Natl Cancer Inst Monogr 66:21–30, 1984

(*18*) LEADON SH, ZOLAN ME, HANAWALT PC: Restricted repair of aflatoxin B_1-induced damage in α-DNA of monkey cells. Nucleic Acids Res 11:5675–5689, 1983

(*19*) ZOLAN ME, SMITH CA, HANAWALT PC: Formation and repair of furocoumarin adducts in α-deoxyribonucleic acid and bulk deoxyribonucleic acid of monkey cells. Biochemistry 23:63–69, 1984

DISCUSSION

D. M. Carter: Dr. Zolan, could you comment on whether there are changes in the state of division or other growth properties of your cells? Do you get the same results if the cells are dividing or if they are in stationary phase?

M. Zolan: I have not done this experiment on rapidly growing cultures; those are a lot harder to do, although they are possible because we use cesium chloride gradients to remove replicated DNA when we want to study repair.

L. E. Bockstahler: I have a question for Dr. Zolan and then a comment. Is this system that you are working with, i.e., α-DNA, applicable with human cells?

Zolan: Oh, yes. There is a homologous sequence in human cells; they are about 65% homologous. Also, as far as we can tell, monkey cells are extremely similar, if not exactly, to human cells with respect to repair.

The human α-sequence has been used by the Haseltine group to study damage. If one wants to isolate pure α-DNA from human cells, it is a lot harder because you can isolate only slightly less than 1% of the DNA as pure α. We are doing repair experiments in human cells, now that we have this form well characterized in monkey cells, to see if we have the same phenomenon. However, because of the way the 2 types of cells behave in culture, I am confident it is a good model system.

K. Kraemer: This actually relates to what Dr. Zolan presented when she mentioned that there is an apparent disparity between repair of the UV damage and repair of the psoralens. This does not surprise me. In some studies with cells from a patient with Cockayne's syndrome, we used lymphoblastoid cells and measured the concentration of viable cells by trypan blue exclusion on successive days after treatment. The untreated cells from a normal subject and the patient with Cockayne's syndrome grew at the same rate. With UVC exposure, though, the normal cells' growth rate slowed down and then increased, whereas the patient's cells were sensitive, and the growth rate failed to increase. The latter cells are hypersensitive to inhibition of growth by the UVC.

We did the same type of experiment using an FS40 artificial sunlamp. Again, the normal and the untreated cells from the patient with Cockayne's syndrome grew at the same rate as each other, but with a dose of this UV, the normal recovered and the Cockayne's syndrome cells never recovered at the low dose. Cells from a patient with this syndrome are hypersensitive to UVC and to this artificial sunlamp. In contrast, when we treated the cells with 8-MOP followed by UVA, the response was identical to the normal response.

The conclusion is that not every cell hypersensitive to UV need be hypersensitive to psoralen; thus psoralen recovery processes may be different from those of UV repair.

Psoralens: A Search for More Effective Derivatives for Photochemotherapeutic Regimens[1, 2]

Isaac Willis *and* Julian M. Menter[3, 4]

ABSTRACT—The objective of our studies under the National Toxicology Program on psoralens was to evaluate a new furocoumarin derivative that would be highly efficacious and yet possess little or no systemic toxicity while also having a short effective biologic half-life. In addition, this work allowed for the development of a test system for compound evaluation of various psoralen derivatives. A guinea pig model was used first, followed by definitive studies in hairless mice for evaluation of the phototoxic potentialities of 32 furocoumarins and 4 benzofuran derivatives. Rank order depends on whether the derivative is administered topically or orally; methyl furocoumarins were the most potent topical photosensitizers, but they were weak when orally administered. On the other hand, aminomethyl derivatives as a group were most potent of all the derivatives when orally administered but were mediocre topical photosensitizers. The standard for these studies, 8-methoxypsoralen (8-MOP), was mediocre in topical and oral regimens. Specific dose-response studies revealed the 5′-aminomethyl-4,4′,8-trimethylpsoralen derivative to be six times more potent than 8-MOP. Moreover, the dose-response curve indicated that the response of the 5′-aminomethyl derivative is eliminated three to four times faster than is 8-MOP. No straightforward relationship between molecular structure and photosensitizing power was found. These results underscore the need for oral and topical evaluation of a given test sensitizer as well as for determination of the chemical nature, temporal distribution, and metabolic fate of its photochemically active form. — Natl Cancer Inst Monogr 66: 143–147, 1984.

Psoralens are furocoumarin compounds that have achieved clinical significance as photosensitizing chemicals efficacious in the treatment of selected dermatologic diseases. They have been prominent in the United States for their role in photochemotherapy (PUVA) of vitiligo (*1, 2*), psoriasis (*3–6*), and to some extent other diseases, such as parapsoriasis, mycosis fungoides (*7–9*), atopic dermatitis, polymorphous light reaction, lichen planus, and pityriasis rosea (*10*). The molecular effects of PUVA include a photochemical modification of DNA due to covalent binding of psoralen molecules to pyrimidine bases resulting in psoralen–DNA cross-links and a subsequent decrease in DNA synthesis (*11*).

Although efficacy of PUVA therapy is exceedingly great (approaching greater than 90% in psoriasis) and can be proved to be more economical (cost-effective) as well as extremely acceptable to the average patient when compared with other forms of therapy (*12, 13*), it is not without significant acute and potential chronic side effects, which include nausea, insomnia, depression, localized blistering, pruritis (*14*), and cataract formation (*15, 16*) in experimental animal studies and in vitro. It also has been shown to induce adverse biologic changes, such as mutagenesis (*17, 18*), carcinogenesis (*19–21*), hyperpigmentation of the skin (*22*), and potential immunologic alterations (*23–32*). Photobinding of the psoralens to nucleic acids is thought to be a major factor responsible for mutagenesis and carcinogenesis (*33*).

Adverse effects are, to a great extent, due primarily to the slow onset of photosensitization or prolonged excretion or degradation of the drug. Development of psoralens in which these characteristics are minimized would be desirable; the ideal psoralen would exhibit rapid and intense photosensitization, rapid apparent excretion from the body, minimal carcinogenic potentiality, and low systemic toxicity. Maximal phototoxicity should occur over a short period and should correspond to the selected period of therapeutic UVA exposure.

The need for a potent, short-lived, less toxic furocoumarin led to the investigation of the above properties of 32 furocoumarin derivatives. In addition, we studied 4 benzofuranyl derivatives thought to undergo cyclization to furocoumarins in vivo. We report here that several of these compounds are more highly phototoxic than is 8-MOP when orally administered and show exceptional promise of fulfilling the "ideal" characteristics cited above.

MATERIALS AND METHODS

Drugs.—All furocoumarins used in this study (table 1, fig. 1) were supplied by Elder Pharmaceuticals (Bryan, Ohio). The commercial solution of 8-MOP (1% Oxsoralen lotion), crystalline 8-MOP, and TMP were used as reference compounds (controls).

Animals.—Both Hartley female albino guinea pigs (700–

Abbreviations: PUVA = psoralen plus UV radiation at 320–400 nm; 8-MOP = 8-methoxypsoralen; TMP = 4,5′,8-trimethylpsoralen; MPD = minimal phototoxic dose(s); J = joule(s).

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[4] We thank Jeanne M. Muzzall, Richard A. Margolin, and Fred B. Setzler for their technical assistance and Ms. Mary G. Perrin for assistance with the manuscript.

TABLE 1.—*Furocoumarin derivatives and benzofuran derivatives used in this study*

Furocoumarin	4	4′	5′	8
I	CH_3	CH_3	CH_2NH_2	CH_3
III	CH_3	H	H	CH_2NH_2
IV	H	H	H	CH_3
V	CH_3	CH_3	CH_2NH_2	H
VI	CH_3	CH_2NH_2	CH_3	CH_3
VIII	H	H	H	Methylene heximinium bromide
IX	CH_3	$HOCH_2CHCH_2NH_2$	CH_3	CH_3
X	CH_3	$HO\,CH_2CH_2\text{-}CH_3$	CH_3	CH_3
XI	CH_3	CH_2NH_2HCl	CH_3	CH_3
XIII	H	CH_2NH_2	H	H
XIV	CH_3	$CH_2COOCH_2CH_3$	CH_3	CH_3
XV	H	CH_3	H	H
XVI	$CH_2CH_2CH_3$	CH_3	H	H
XVII	H	CH_3	H	CH_3
XVIII	H	Methoxyphenyl	H	H
XIX	CH_3	CH_2NH_2	H	H
XX	CH_3	CH_2COOH	CH_3	CH_3
XXI	CH_3	CH_3	H	CH_3
XXIII	H	Chlorophenyl	H	H
XXIV	H	Phenyl	H	H
XXV	CH_3	CH_3	H	H
XXVI	H	CHO	H	CH_3
XXVII	H	H	H	CH_2NH_2
XXVIII	CH_3	$Cl^-(CH_3)^+N\,CH_2$	CH_3	CH_3
XXIX	CH_3	CH_3	CH_2CN	CH_3
XXX	CH_3	*N*-pyridinium methylchloride	CH_3	CH_3
XXXI	H	CH_3	H	$CH_2{=}CH{-}CH_2$
XXXII	H	CH_3	CH_2NH_2	H
XXXIII	H	H	CH_3	CH_3
8-MOP	H	H	H	OCH_3
Tripsoralen	CH_3	H	CH_3	CH_3
Benzofuran	R_1	R_2	R_3	R_4
II	H	H	H	CH_3
VII	CH_3	H	CH_3	CH_3
XII	H	CH_3	H	CH_3
XXII	H	CH_3	H	H
XXXIV (Angelicin)				

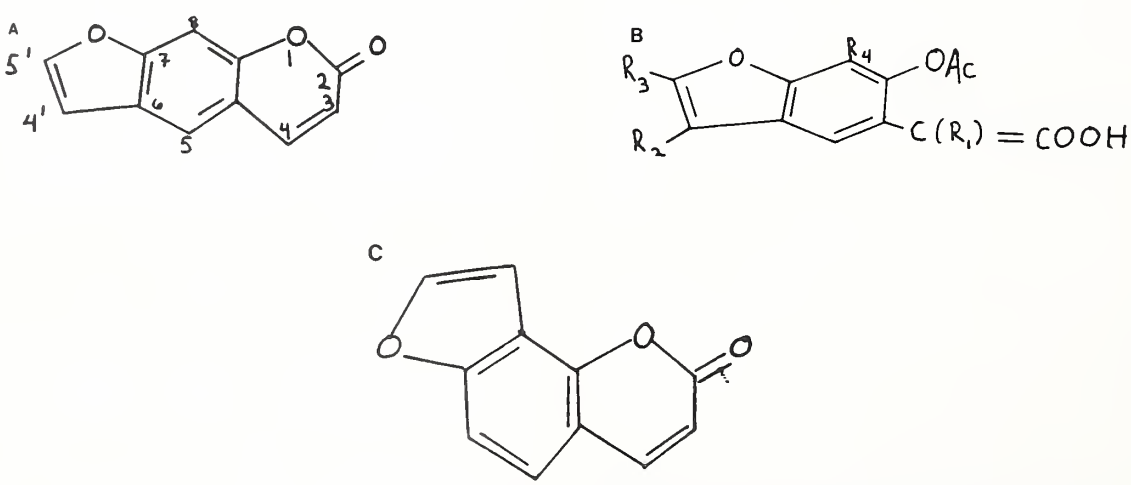

FIGURE 1.—Structural formulas of the furocoumarin (A) and benzofuran derivatives (B) and angelicin (C).

1,000 g) and albino hairless mice (SK-1 and HRA/Skh strains weighing about 30 g) were used. All animals were housed in a holding cage without food or water for 4 hours before drug administration and were weighed immediately before gavage. The mid-dorsal region of the back of each guinea pig was shaved with electric clippers. A commercial depilatory (Nair), applied to the shaved area for 10 minutes and then rinsed away with warm water, left the skin smooth and free of residue. Duplicate tests were made with wax as an epilating material. Any animal having adverse reactions to the depilatories (such as abrasions or irritant reactions) was not used for further experimentation. The epilated areas were covered with templates made from double-backed adhesive tape. The skin was exposed through 0.25- or 0.5-cm^2 squares cut into the templates. The unexposed areas were protected from irradiation with black contact paper.

Oral administration and testing of drugs.—Guinea pigs fasted as previously described were given gelatin capsules [size 5; Eli Lilly & Co. (Indianapolis, Ind.)] packed with 2, 5, 10, 20, or 40 mg furocoumarin/kg body weight. Administration of the capsule was followed by a rinse of 1 ml tap water. For hairless mice, appropriate amounts of drug were mixed with 0.1 ml (0.091 g) melted, clarified unsalted butter that was sonicated and then administered to the mouse through a tuberculin syringe equipped with a stainless steel mouse feeding needle obtained from Popper & Sons, Inc. (New Hyde Park, N.Y.).

We then exposed designated sites on the skin to UVA radiation at varying but standard intervals from 10 to 300 minutes after gavage to determine the onset, duration, and peak of photosensitization.

Light source and exposure.—The animals were irradiated at a distance of 12 cm from a bank of UVA lamps, the output of which at 12 cm was 9.3 milliwatts/cm^2. Total flux energy output was determined with a radiometer, Model IL-442A obtained from International Light Co. (Newburyport, Mass.), and calibrated against a lamp traceable to the United States Bureau of Standards. The UV spectral output of the lamp peaks at 360 nm with insignificant radiation output shorter than 320 nm and longer than 420 nm. Irradiation doses were as indicated in the tables. Following irradiation, the square areas were marked at the edges with black ink, and the templates were removed. Except for the short periods of visual examination, the animals were kept away from direct lighting for the next 72 hours. They were examined for erythemic responses at 24, 48, and 72 hours. Control animals were exposed to the same doses of light in the absence of drug.

Minimal phototoxic dose determination.—We evaluated selected furocoumarins to determine the MPD of furocoumarin. In these tests, the drug dosage was held constant at 10 mg/kg, but the dose of UVA radiation was varied. The MPD was defined as that amount of irradiation which induced a minimal (i.e., barely perceptible) erythema of skin extending to the margins of the test site. Animals were exposed to UVA at the time of maximal skin responses as determined above (*see* table 2).

TABLE 2.—*Erythemic activity of selected furocoumarin derivatives after oral dosages*[a]

Furocoumarin derivative	Oral dose, mg/kg	Erythemic response after minutes of exposure:									
		10	20	30	45	60	90	120	130	240	300
I	40	2+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	10	0	1+	3+	3+	4+	4+	4+	3++	3+	3+
	5	0	0	1+	3+	4+	4+	2+	±	0	0
	2	0	0	0	2+	3+	2+	0	0	0	0
II	40	2+	2+	3+	4+	4+	4+	4+	4+	4+	3+
	10	2+	2+	3+	3+	4+	4+	4+	3+	1+	0
	5	1+	1+	1+	2+	2+	4+	1+	0	0	0
III	40	0	1+	1+	4+	4+	4+	1+	1+	1+	1+
	10	0	0	1+	2+	2+	4+	4+	3+	1+	0
IV	40	0	0	2+	3+	4+	4+	3+	4+	1+	1+
	10	0	0	0	2+	2+	4+	2+	1+	0	0
V	40	0	3+	4+	4+	4+	4+	3+	2+	1+	±
	10	0	1+	1+	2+	2+	2+	4+	1+	0	0
VI	40	±	1+	3+	4+	4+	2+	2+	2+	2+	2+
	10	0	1+	2+	3+	3+	3+	3+	3+	2+	2+
8-MOP	40	0	0	1+	3+	3+	3+	4+	3+	2+	2+
	10	0	0	0	1+	1+	2+	2+	1+	0	0
Tripsoralen	40	0	0	1+	1+	0	0	0	0	0	0
Angelicin XXXIV	40	0	0	0	0	0	0	0	0	0	0

[a] Dosage of UVA light was 1.14 J/cm^2. 0=no response; ±=faint erythema; 1+=erythema; 2+=intense erythema; 3+=erythema and edema; 3++=erythema and intense edema; and 4+=vesiculobullous reaction.

Gradation of erythema.—Erythema responses for topical and oral dosage tests were evaluated with the use of the following criteria:

Grade	Criteria
±	Faint barely discernible erythema
1+	Mild erythema, easily discernible from adjacent skin
2+	Marked area of red coloration
3+	Intense erythema with moderate edema
3++	Severe erythema, extensive edema
4+	Erythema, edema with vesiculobullous reaction

RESULTS

Only 6 derivatives (compounds I-VI) gave reactions equal to or more intense than 8-MOP (table 2). The degree of erythemic activity varied with the amounts of drug administered. For compounds I–V, the responses at 40 mg/kg were so severe that it was impossible for us to determine the peak *reaction time* because 4+ responses were obtained over a wide range or period (table 2). By decreasing the drug dosage, we could determine a more precise time course dose–response curve. Compound I proved to be the most potent compound; 4+ responses were elicited with as little as 2 mg/kg, whereas similar or less intense responses with other compounds required higher drug doses. Six other derivatives (compounds VII–XII) produced moderate to severe erythemic responses, but these were not as intense as those elicited by 8-MOP under similar test conditions. The remaining compounds, including trisoralen and angelicin, produced extremely poor erythema responses.

Lowering the dosage from 40 to 10 mg/kg caused an apparent shifting of peak activity for compounds III, V, and VI (table 2). Observations further revealed that at drug doses high enough to evoke a 4+ response, compounds I, II, and V had early onset of photosensitization in comparison to the standard 8-MOP.

The MPD of several of the more phototoxic compounds, i.e., compounds I, II, III, and XI, were compared with the MPD of 8-MOP determined under similar conditions.

TABLE 3.—*MPD of selected furocoumarins*[a]

Furocoumarin derivative	Time irradiated after psoralen dose, min[b]	Minimal phototoxic UVA dosage, J/cm^2	
		Guinea pigs	Hairless mice
I	90	0.102–0.130	0.15–0.19
II	"	0.19 –0.206	
III	"	0.230–0.267	
VI	120	0.305–0.325	
8-MOP	"	0.651–0.697	0.95–1.25

[a] The MPD of UVA are those dosages of light that elicit a minimal erythemic response to the skin (1+).

[b] Psoralens were administered at a dosage of 10 mg/kg body weight, and animals were exposed to UVA at the times at which responses were maximally observed in the skin.

Most notably, 5′-aminomethyl-4,4′8-trimethyl derivative (I) was six times more potent than was 8-MOP, based on MPD responses following oral administration, and was roughly two and three times as potent as compounds III and VI, respectively (table 3), which in turn were two to three times as potent as 8-MOP by this criterion.

DISCUSSION

One of our primary objectives of this study has been to identify compounds which show promise as potent photochemotherapy agents while possessing low systemic toxicity and short effective biologic half-life. In this work, we have used cutaneous erythema as the criterion on which to rank the photosensitizing capabilities of 32 furocoumarins and 4 benzofuranyl derivatives. The rank order depends on whether oral or topical administration is used [Willis I, Muzzall JM, Menter JM: Unpublished results; *see* also (*33*)]. The methyl derivatives were the most potent topical photosensitizers (*33*) but were weak when administered orally. On the other hand, the aminomethyl derivatives as a group were *more* potent oral photosensitizers than was 8-MOP, although mediocre (Willis I, Muzzall JM, Menter JM: Unpublished observations) when applied topically. Such dependence of photosensitizing potency on the mode of administration has been previously discussed by Pathak et al. (*33*). Some of the reasons for the observed differences derive from 1) in vivo absorption and resulting blood and epidermal levels, 2) metabolism and rate of biotransformation to either active or inactive moieties, and 3) rate of excretion (for oral administration) as well as concentration solubility and rate of diffusion through the stratum corneum (for topical administration). In the absence of detailed information on the above parameters, we cannot presently establish the relative importance of each of the above phenomena for the series of compounds studied here. With regard to the last point, a direct comparison of the "inherent" sensitizing activity is difficult because the widely varying solubility of these compounds makes it necessary for several solvents to be used within the series.

Inspection of this series of compounds reveals no straightforward relationship between structure and photosensitizing power. Perhaps such a result might have been expected, inasmuch as the major properties which determine photosensitizing capability are *excited-state* characteristics (which are often distinctly different from those of the ground state), mode of administration (*33*), as well as the potential ability to undergo biotransformation to a more potent sensitizer in vivo.

In conclusion, the major impact of our preliminary screening was the revealing of a number of active furocoumarins which show promise of serving as active oral photochemotherapy agents. These agents have a rapid time course of photosensitivity and appear to have low systemic toxicity. Moreover, small doses are required to elicit potent phototoxic reactions. These results underscore the need for both oral and topical evaluation of a given sensitizer, determination of the nature and temporal distribution of the active forms, as well as the rate of absorption, excretion, and transformation under oral and topical distribution. More chemical information is needed for these

compounds, so that a theoretically more reasonable structure–function relationship can be made.

REFERENCES

(*1*) Lerner AB, Denton CR, Fitzpatrick TB: Clinical and experimental studies with 8-methoxypsoralen in vitiligo. J Invest Dermatol 20:299–314, 1953

(*2*) Arora SK, Willis I: Factors influencing methoxsalen phototoxicity in vitiliginous skin. Arch Dermatol 112: 327–332, 1976

(*3*) Parrish JA, Fitzpatrick TB, Tanenbaum L, et al: Photochemotherapy of psoriasis with oral methoxsalen and longwave ultraviolet light. New Engl J Med 291:1207–1211, 1974

(*4*) Melski JW, Tanenbaum L, Parrish JA, et al: Oral methoxsalen photochemotherapy for the treatment of psoriasis: A cooperative clinical trial. J Invest Dermatol 68:328–335, 1977

(*5*) Roenigk HH Jr: Photochemotherapy for psoriasis: A clinical cooperative study of PUVA-48 and PUVA-64. Arch Dermatol 115:576–579, 1979

(*6*) Vella Briffa D, Greaves MW, Warin AP, et al: Relapse rate and long-term management of plaque psoriasis after treatment with photochemotherapy and dithranol. Br Med J 282:937–940, 1981

(*7*) Gilchrest BA, Parrish JA, Tanenbaum L, et al: Oral methoxsalen photochemotherapy of mycosis fungoides. Cancer 38:683–689, 1976

(*8*) Roenigk HH Jr: Photochemotherapy for mycosis fungoides. Arch Dermatol 113:1047–1051, 1977

(*9*) DuVivier A, Vollum DI: Photochemotherapy and topical nitrogen mustard in the treatment of mycosis fungoides. Br J Dermatol 102:319–322, 1980

(*10*) Song P-S, Tapley KJ Jr: Photochemistry and photobiology of psoralens. Photochem Photobiol 29:1777–1797, 1979

(*11*) Musajo L, Rodighiero G, Caporale G, et al: Photoreactions between skin-photosensitizing furocoumarins and nucleic acids. *In* Sunlight and Man: Normal and Abnormal Photobiologic Responses (Fitzpatrick TB, Pathak MA, Harber LC, et al, eds). Tokyo: Univ Tokyo Press, 1974, pp 369–387

(*12*) Stern RS, Thibodeau LA, Kleinerman RA, et al: Effects of methoxsalen photochemotherapy on cost of treatment for psoriasis. JAMA 245:1913–1918, 1981

(*13*) Roenigk HH Jr: Cost-effectiveness of PUVA. JAMA 245: 1941, 1981

(*14*) Wolff K: Photochemotherapy of psoriasis (PUVA). *In* Research in Photobiology (Castellani A, ed). New York: Plenum Press, 1977, pp 409–417

(*15*) Cloud TM, Hakim R, Griffin AC: Photosensitization of the eye with methoxsalen. II. Chronic effects. Arch Ophthalmol 66:689–694, 1961

(*16*) Freeman RG, Troll D: Photosensitization of the eye by 8-methoxypsoralen. J Invest Dermatol 53:449–453, 1969

(*17*) Vella Briffa D, Warin AP: Photochemotherapy in psoriasis: A review. J R Soc Med 72:440–446, 1979

(*18*) Bridges B, Strauss G: Possible hazards of photochemotherapy for psoriasis. Nature 28:523–524, 1980

(*19*) Stern RS, Thibodeau LA, Kleinermann RA, et al: Risk of cutaneous carcinoma in patients treated with oral methoxsalen photochemotherapy for psoriasis. New Engl J Med 300:809–813, 1979

(*20*) Halprin KM: Psoriasis, skin cancer and PUVA. J Am Acad Dermatol 2:334–337, 1980

(*21*) Roenigk HH Jr, Caro WA: Skin cancer in the PUVA–48 cooperative study. J Am Acad Dermatol 4:319–324, 1981

(*22*) Parrish JA, Pathak MA, Fitzpatrick TB: Prevention of unintentional overexposure to topical psoralen treatment of vitiligo. Arch Dermatol 104:281–283, 1971

(*23*) Morison WL: Photoimmunology. J Invest Dermatol 77: 71–76, 1981

(*24*) Volden G, Molin L, Thomsen K: PUVA-induced suppression of contact sensitivity to mustine hydrochloride in mycosis fungoides. Br Med J 2:865–866, 1978

(*25*) Thoravaldesen J, Volden G: PUVA-induced diminution of contact allergic and irritant skin reactions. Clin Exp Dermatol 5:43–46, 1980

(*26*) Morison WL, Parrish JA, Woehler ME: The influence of ultraviolet radiation on allergic contact dermatitis in the guinea-pig. II. Psoralen/UVA radiation. Br J Dermatol 104:165–168, 1981

(*27*) Halprin KM, Comerford M, Presser SE: Ultraviolet light treatment delays contact sensitization to nitrogen mustard. Br J Dermatol 105:71–76, 1981

(*28*) Morison WL, Parrish JA, Bloch KJ, et al: Transient impairment of peripheral blood lymphocyte function during PUVA therapy. Br J Dermatol 101:391–397, 1979

(*29*) Friedmann PS, Rogers S: Photochemotherapy of psoriasis: DNA damage in blood lymphocytes. J Invest Dermatol 74:440–443, 1980

(*30*) Morhenn VB, Benike CJ, Engleman EG: Inhibition of cell-mediated immune responses by 8-methoxypsoralen and long-wave ultraviolet light: A possible explanation for the clinical effects of photoactivated psoralen. J Invest Dermatol 75:249–252, 1980

(*31*) Morison WL, Parrish JA, Bloch KJ, et al: In vivo effects of PUVA on lymphocyte function. Br J Dermatol 104:405–413, 1981

(*32*) Vella Briffa D, Parker D, Tosca N, et al: The effect of photochemotherapy (PUVA) on cell-mediated immunity in the guinea pig. J Invest Dermatol 77:377–380, 1981

(*33*) Pathak MA, Fitzpatrick TB, Parrish JA, et al: Photochemotherapeutic, photobiological, and photochemical properties of psoralens. *In* Research in Photobiology (Castellani A, ed). New York: Plenum Press, 1977, pp 267–279

DISCUSSION

M. Kripke: Dr. Willis, it seems to me that 1) you are using numbers of milligrams/kilogram body weight in all of these studies, and 2) the molecular weight of each compound is going to be quite different depending on what the substitution is.

J. Menter: I can tell you that the variation was less than 20% in the molecular weights of all the compounds.

M. A. Pathak: As much as I like this kind of presentation, I find a couple of deficiencies in this type of system in which the compound is being administered in capsules. It is important to have it in a solution, so that at least the absorption question is minimal. One problem that arises is that the guinea pig can keep the capsule somewhere in a pouch and spit it out later.

Induction of Sister Chromatid Exchanges and Gene Mutations in Chinese Hamster Ovary Cells by Psoralens[1, 2]

Kenneth S. Loveday *and* Brian A. Donahue[3, 4]

ABSTRACT—Three linear psoralen compounds, 8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP), 3-carbethoxypsoralen (3-CP), and one angular psoralen, 5-methylangelicin (5-ANG), were tested for their ability to induce both sister chromatid exchanges (SCE) and gene mutations (*hypoxanthine-guanine phosphoribosyltransferase* locus) in vitro in Chinese hamster ovary cells (CHO line). All the compounds induced both SCE and mutations in the presence of UV irradiation (UVA; peak at 330–380 nm), but no increases were observed in its absence. The frequency of both responses increased with either 1) increasing concentration of compound with a fixed amount of UVA or 2) increasing amount of UVA with a fixed concentration of psoralen. Significant increases in SCE were seen for 8-MOP, 5-MOP, and 5-ANG at concentrations near 1×10^{-6} *M*, whereas concentrations near 20×10^{-6} *M* of 3-CP were needed before increases in SCE were observed. The induction of gene mutations followed a similar pattern; concentrations of $50–100 \times 10^{-6}$ *M* of 3-CP were needed to induce large increases in mutations, but much lower concentrations of 8-MOP, 5-MOP, and 5-ANG ($5–10 \times 10^{-6}$ *M*) were sufficient to induce large increases in mutations. The ratio of induced mutations to induced SCE was similar for 8-MOP, 5-MOP, and 3-CP; that of 5-ANG was much higher, which indicated that the linear furocoumarins produce a different spectrum of DNA damage from that produced by the angular psoralen. — Natl Cancer Inst Monogr 66: 149–155, 1984.

Because PUVA is used to treat patients with psoriasis as well as other diseases (*1–3*), considerable research has focused on understanding the photobiologic properties of these molecules and the underlying mechanism of this kind of therapy. The report that 8-MOP plus UVA can lead to an increase in skin cancer in patients (*4*) has prompted further interest in designing the most effective and yet safest PUVA treatment.

Mutations are induced by PUVA in bacteria (*5–8*), yeast (*9–11*), fungi (*12, 13*), and in mammalian cells (*8, 14–17*), and also SCE are induced in vitro in mammalian cells (*5, 18–23*) and in vivo in the hamster cheek pouch (*24*). The importance of determining the effect of PUVA in these in vitro and in vivo systems is based on the correlation between induction of either SCE or mutations in these test systems and carcinogenesis in animals or man (*25–28*). Consistent with this idea are the observations that PUVA (8- and 5-MOP in conjunction with UVA) induces gene mutations and SCE and also induces tumors in mice (*29, 30*).

In our study reported here, the ability of 4 psoralen compounds, 8-MOP, 5-MOP, 3-CP, and 5-ANG, to induce both SCE and gene mutations (*HGPRT* locus) in the CHO cell line was determined. Our goals were to 1) determine whether any differences occur between linear and angular psoralens, 2) determine whether any differences exist between psoralen compounds which can cause cross-links in DNA and those which presumably cause only monoadducts, and 3) provide data to determine which of the end points, either SCE or mutations, correlates better with efficacy in PUVA therapy and induction of tumors in either man or animals.

MATERIALS AND METHODS

Cell culture.—The CHO cells were obtained from Dr. Samuel A. Latt, Harvard Medical School (Boston, Mass.), and routinely grown in modified Ham's F10 medium as described in (*21*). We routinely screened them for the presence of mycoplasma at Bioassay Systems using the fluorescent dye, 4′,6-diamidino-2-phenylindole dihydrochloride. Cell culture medium for mutagenesis experiments was F12 without hypoxanthine and supplemented with 5% dialyzed fetal calf serum.

Mutagenesis assay.—The procedure for mutagenesis closely followed methods developed by O'Neill and associates (*31, 32*). Cells grown in F10 medium were exposed to varying conditions of PUVA as described in the text. Following removal of the psoralen, the cells were washed several times with either saline or medium, and then were detached with the use of trypsin. They were counted, and aliquots were plated in P100 dishes for cell survival determinations; the remainder was subcultured at approximately 1×10^6 per T75 flask every 2–3 days for the expression time. After 7–8 days, the toxicity plates were stained. After a minimum of 8 days, the remaining cells were selected for the presence of HGPRT mutants by plating of 2×10^5 cells per P100 dish in F12 medium

ABBREVIATIONS: PUVA = psoralen plus UV radiation at 320–400 nm; 8-MOP = 8-methoxypsoralen; SCE = sister chromatid exchange(s); 5-MOP = 5-methoxypsoralen; 3-CP = 3-carbethoxypsoralen; 5-ANG = 5-methylangelicin; HGPRT = hypoxanthine-guanine phosphoribosyltransferase; CHO = Chinese hamster ovary; J = joule(s); mW = milliwatt(s); BrdUrd = 5-bromodeoxyuridine.

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[3] Bioassay Systems Corporation, 225 Wildwood Avenue, Woburn, Massachusetts 01801.

[4] We thank Ms. Marybeth Thomas and Ms. Monica Norman for their technical assistance and Dr. Samuel A. Latt for his helpful discussions.

containing 5% dialyzed fetal calf serum, no hypoxanthine, and 2 μg 6-thioguanine/ml. Then 1.2×10^6 cells were selected from each flask. Concomitantly with the selection step, we plated 200 cells from each flask in medium without 6-thioguanine to determine the cloning efficiency. All conditions were tested in duplicate for both mutagenesis and toxicity.

By using the cloning efficiency of the selected cells, we calculated the mutation frequency. Ethyl methanesulfonate was the positive control in the mutagenesis experiments. Mutation frequencies typically were 250×10^{-6} for 300 μg/ml (data not shown).

Cytogenetic procedures.—We plated the CHO cells approximately 1 day before exposure to PUVA in F10 medium. The appropriate amounts of psoralen were added 1 hour before irradiation. Following exposure to UV, the cells were washed several times and then grown for 28–30 hours in medium containing 2×10^{-5} *M* BrdUrd. This time was slightly longer than that needed for detection of SCE in control cultures, but the treatment of the cells with high concentrations of psoralen and high doses of UV often delayed the cycling of the cells. Delay in the time of harvest caused an increase in the number of cells with differentiated sister chromatids. Approximately 2 hours before cell harvest, vinblastine sulfate was added at a final concentration of 0.25 μg/ml. Cells were heated for 12 minutes at 37° C in hypotonic solution (0.03 *M* potassium chloride, 0.01 *M* sodium citrate) and then centrifuged and fixed three times with methanol:acetic acid (3:1). Slides were prepared and stained according to the method of Perry and Wolff (*33*) as described in (*34*), with the modification that slides stained with 33258 Hoechst were illuminated for 30 minutes by a PUVA lamp instead of a white fluorescent light (which requires several hours of illumination). Subsequent washing in saline and Giemsa staining followed methods we had used earlier (*34*). Cells with differentially stained sister chromatids were photographed, and SCE were scored from photographic prints.

Chemicals.—The psoralen compounds, 8-MOP, 5-MOP, 3-CP, and 5-ANG, were dissolved in methanol immediately before use. Subsequent dilutions were made with 50% methanol and then cell culture medium.

Irradiation conditions.—Lamps from GTE Sylvania–Emissive Products (#FR40T12/PUVA; Exeter, N.H.) were used for UV irradiation. The peak of irradiation was between 330 and 380 nm.

Cells were irradiated in plastic tissue culture flasks placed approximately 20 cm from 2 PUVA lights. The intensity was measured immediately before irradiation and was approximately 1.3 mW/cm^2 (1 $mW/cm^2 = 10\ J \cdot m^{-2} \cdot second^{-1}$); times of irradiation were adjusted to yield the desired doses of UV and typically ranged from 75 to 225 seconds. Intensity was monitored with an International Light, Inc. IL700A radiometer (Newburyport, Mass.) with the UVA-probe SEE015/UVA/W/P112.

RESULTS

Induction of Sister Chromatid Exchanges

The 4 psoralen compounds were tested for their ability to induce SCE under conditions in which either the UV dose was increased with a fixed amount of psoralen or the concentration of psoralen was increased with a fixed amount of UV irradiation. Neither psoralen in the absence of UV irradiation nor UVA without psoralen induced SCE (figs. 1, 2; tables 1, 2).

Under nearly equimolar conditions, all 4 compounds yielded increases in the average number of SCE/cell as the UV dose was increased (fig. 1). Both 5-MOP and 8-MOP produced nearly linear increases in the number of SCE/cell with increasing UV dose. The increase in SCE produced by 3-CP under these conditions was minimal. The induction of SCE by 5-ANG reached a plateau of 35–40 SCE/cell at 0.5 to $1 \times 10^3\ J/m^2$, whereas the induction of SCE by 5-MOP and 8-MOP continued to increase to the limits of the experiment (2.5 to $3.0 \times 10^3\ J/m^2$).

These compounds yielded increases in SCE/cell as the concentration of psoralen was increased but with a constant amount of UV irradiation at $1 \times 10^3\ J/m^2$ (figs. 2, 3). Both 5-MOP and 8-MOP produced almost linear increases, but 5-ANG again yielded a curve which reached a plateau at low concentrations, $1–2 \times 10^{-6}$ *M*, compared with either 5-MOP or 8-MOP. Higher concentration of 5-ANG, 30 or 100×10^{-6} *M*, prevented cells from growing for 2 cycles, and sister chromatid differentiation was not observed. The induction of SCE by 3-CP required much higher concentrations, from 20 to 100×10^{-6} *M*, compared with the other 3 compounds (fig. 3). Given a sufficiently high concentration, 100×10^{-6} *M*, and sufficient UV irradiation, 1.5 or $3.0 \times 10^3\ J/m^2$, we obtained SCE levels comparable to those with both 5-MOP and 8-MOP, i.e., 60 SCE/cell. The combination of 300×10^{-6} *M* 3-CP with $3 \times 10^3\ J/m^2$ prevented the appearance of cells with differentiated sister chromatids.

Induction of Sister Chromatid Exchanges and Gene Mutations

Based on the information obtained in the SCE experiments, further studies were performed in which toxicity, induction of SCE, and induction of $HGPRT^-$ mutations

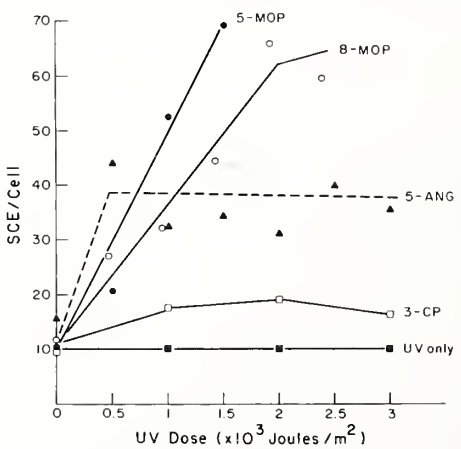

FIGURE 1.—Dependence of induction of SCE on UV dose by 8-MOP (*open circles*), 5-MOP (*closed circles*), 3-CP (*open squares*), and 5-ANG (*closed triangles*). Cells exposed only to UV are represented by the *closed squares*. Psoralen concentration was 3×10^{-6} *M* for 5-MOP, 3-CP, and 5-ANG, and 4×10^{-6} *M* for 8-MOP.

TABLE 1.—*Induction of mutations and SCE by 8-MOP and 5-MOP*[a]

Compound	Concentration, 10^{-6} *M*	UV dose, 10^3 J/m^2	Percent survival[b]	Mutation frequency, $\times 10^{-6}$	No.	$\bar{X}$	SCE No.	SCE $\bar{X}$	Total No.	Total $\bar{X}$
8-MOP	0	0	100	5.3	14	9.6				
	0	1.0	113	2.0	12	9.8				
	0	2.5	137	1.8	13	7.5				
	4	0	136	7.4	11	7.7				
	4	1.0	117	19.6	29	34.2	21	32.3	50	33.4
	4	2.5	62	68.4	12	63.8	18	59.2	30	61.0
	10	0	134	3.7	7	9.7				
	10	1.0	76	50.2	13	56.8	28	63.6		
							13	63.3	54	61.9
	10	2.5	3	351	No second division cells					
	20	0	120	4.3	ND					
	20	1.0	79	106	ND					
	20	2.5	0.3	No cells	ND					
5-MOP	0	0	100	9.2	15	8.8				
	0	1.5	133	13.4	10	9.6				
	0	3.0	92	21.7	13	10.2				
	1.5	0	92	25.4	14	9.1				
	1.5	1.5	122	ND	29	34.0				
	1.5	3.0	90	52.5	21	49.3				
	3.0	0	187	<1.2	15	10.3				
	3.0	1.5	150	59.2	18	52.5	21	69.1	39	61.4
	3.0	3.0	15	189	No second division cells					
	4.0	0	124	17	ND					
	4.0	1.5	65	112	ND					
	4.0	3.0	47	90	ND					

[a] The SCE data from the experiment conducted at the same time as the mutagenicity experiment are shown in the left-hand column under the heading, "SCE." Data from other experiments are shown next, and an average of 2 or more experiments is shown under "Total." "No second division cells" means that no metaphase cells with differentially stained sister chromatids were present. ND = not determined.

[b] Percent survival is expressed relative to the cloning efficiency of the unexposed cells (no psoralen, no UV).

were all measured under identical experimental conditions (tables 1, 2).

The 4 compounds induced mutations at the *HGPRT* locus in CHO cells that followed a similar pattern to the induction of SCE previously reported; much higher concentrations of 3-CP were needed for a detectable increase in mutations. The plateau in the induction of SCE by 5-ANG, however, was not observed for the induction of mutations. Using conditions which caused a plateau in SCE/cell for 5-ANG, we obtained further increases in the mutation frequency (table 2). For 5-MOP, 8-MOP, and 3-CP, increases in the mutation frequency were obtained under

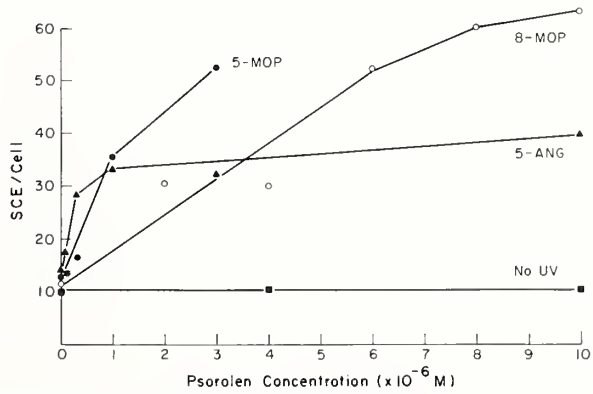

FIGURE 2.—Dependence of induction of SCE on psoralen concentration by 8-MOP (*open circles*), 5-MOP (*closed circles*), and 5-ANG (*closed triangles*). Cells exposed to 8-MOP without UV are represented by *closed squares*. The UV dose was 1×10^3 J/m^2 for 5-MOP and 5-ANG and 0.96×10^3 J/m^2 for 8-MOP.

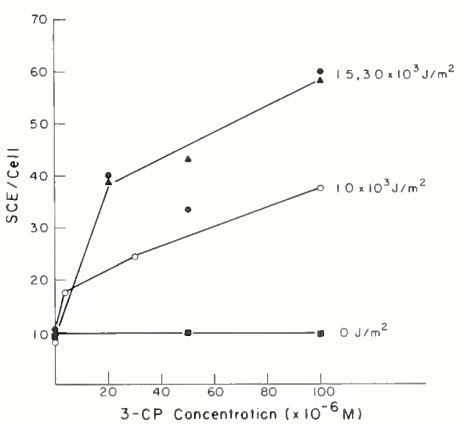

FIGURE 3.—Induction of SCE by 3-CP and UV (dependence on concentration). Symbols: 0 J/m^2, *closed squares;* 1×10^3 J/m^2, *open circles;* 1.5×10^3 J/m^2, *closed circles;* 3.0×10^3 J/m^2, *closed triangles.*

TABLE 2.—*Induction of mutations and SCE by 3-CP and 5-ANG*[a]

Compound	Concentration, 10^{-6} *M*	UV dose, 10^3 J/m^2	Percent survival[b]	Mutation frequency, $\times 10^{-6}$	No.	$\bar{X}$	SCE		Total	
							No.	$\bar{X}$	No.	$\bar{X}$
3-CP	0	0	100	3.1	26	6.9				
	0	1.5	156	2.0	29	8.3				
	0	3.0	109	2.7	26	9.6				
	50	0	105	4.2	28	7.3				
	50	1.5	96	19.5	24	33.5				
	50	3.0	43	53.0	16	43.3				
	100	0	93	2.2	29	8.8				
	100	1.5	49	85.9	27	60.4	13	64.1	40	61.6
	100	3.0	22	63.7	14	58.5				
	150	0	ND	<2.0	ND					
	150	1.5	ND	90.7	ND					
	150	3.0	ND	105	ND					
5-ANG	0	0	100	22.8	26	9.8				
	0	1.5	114	4.7	28	10.2				
	0	3.0	94	6.4	30	8.7				
	5	0	108	6.4	29	8.7				
	5	1.5	114	103	29	23.1				
	5	3.0	134	184	30	33.5				
	10	0	100	8.6	27	9.1				
	10	1.5	106	176	13	19.8				
	10	3.0	96	141	27	39.3				
	15	0	89	24	ND					
	15	1.5	94	220	ND					
	15	3.0	69	370	ND					

[a] *See* footnote *a*, table 1.
[b] *See* footnote *b*, table 1.

conditions in which sister chromatid differentiation was not observed. By increasing either the psoralen concentration or the dose of UV, we created conditions in which cell survival was low enough to prevent sufficient cells from growing for 2 cycles in BrdUrd (conditions necessary to achieve sister chromatid differentiation), but enough cells survived the treatment so that the mutagenesis experiment could be completed. Large increases in the mutation frequency, comparable to those obtained with 5-ANG, were observed only with conditions which resulted in significant cell death (as low as 3–4% survival sometimes). Appreciable increases in the mutation frequency were observed with 5-ANG under conditions in which there was little or no cell toxicity.

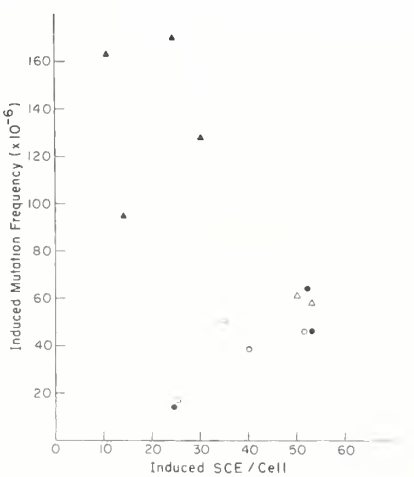

FIGURE 4.—Relationship of induced SCE to induced mutation frequency for 8-MOP, 5-MOP, 3-CP, and 5-ANG. Results are plotted for conditions in which both SCE and HGPRT$^-$ mutants were measured. Data are taken from tables 1 and 2. Background values for SCE and mutation frequency were subtracted for this figure. 5-ANG, *closed triangles;* 3-CP, *open triangles;* 8-MOP, *closed circles;* 5-MOP, *open circles.*

We also measured SCE for each compound using cells exposed to identical conditions at the same time the mutagenesis experiments were conducted. These SCE frequencies are also recorded in tables 1 and 2; the results of these SCE experiments are consistent with those shown in figures 1–3. Under conditions which resulted in significant cell death, no cells with differentiated sister chromatids were observed.

To compare the results of the SCE and the mutagenesis experiments for the 4 compounds, we replotted the data (fig. 4). For a given set of PUVA conditions, the number of induced mutations was plotted versus the number of induced SCE (background values were subtracted for both SCE and mutation frequency). The points fall into 2 relative classes. The data for 3-CP, 5-MOP, and 8-MOP are nearly identical and are characterized by a low ratio of induced mutations per induced SCE. Large increases in SCE, over 50/cell, were accompanied by only modest increases in the mutation frequency, 50 to 60 mutants/10^6 survivors. Further increases in the mutation frequency were obtained with these compounds, but these points are not

shown on this graph because cells with differentiated sister chromatids were not observed under identical conditions.

In contrast, 5-ANG is characterized by a high ratio of induced mutations:induced SCE. Under conditions in which only 10 or 20 SCE were induced, large increases in mutation frequency were seen, i.e., 100 to 150 mutants per 10^6 survivors.

DISCUSSION

The data presented here demonstrate that 8-MOP, 5-MOP, 3-CP, and 5-ANG in conjunction with UVA can induce both SCE and gene mutations in CHO cells. The data for 8-MOP are consistent with previous results for the induction of SCE in CHO cells (*21*) and for the induction of $HGPRT^-$ mutations in either CHO cells (*16*) or Chinese hamster lung cells, V79 (*8, 15, 17*). The data for 5-MOP are also consistent with previous results for the induction of SCE in CHO cells (*5*), although the PUVA conditions were altered. We used lower concentrations of 5-MOP, 3×10^{-6} *M* compared with 200×10^{-6} *M*, and higher doses of UV light, 3×10^3 J/m^2 compared with 0.6×10^3 J/m^2, to obtain similar increases in SCE. The induction of mutations by 5-ANG, tested at similar concentrations to those previously reported (*8*), produced similar results. Pani et al. (*8*) reported that 5-ANG produced the same number of mutants as 8-MOP, and our mutation results are consistent with their observation.

Much higher concentrations of 3-CP compared with either 5-MOP, 8-MOP, or 5-ANG, were needed to induce either SCE or mutations. The data presented by Isaacs et al. (*35*) that the binding of 3-CP to purified DNA after UV irradiation is approximately 2% of the binding of 5-MOP, 8-MOP, or 5-ANG to DNA explains the need for higher concentrations of 3-CP for one to obtain increases in SCE and mutations in CHO cells. The lower binding affinity of 3-CP to isolated DNA reported by Isaacs and co-workers is consistent with that reported by Averbeck and associates (*36*) for 3-CP and isolated DNA, although Averbeck also reported that 3-CP bound more tightly to yeast DNA in vivo than did 8-MOP. The reason for this difference between in vitro and in vivo binding is not known.

The induction of SCE by 5-ANG, the only angular psoralen assayed, differed from the SCE induction pattern obtained with the 3 linear psoralens. After a sharp increase in SCE over a narrow range, based either on increasing concentration or on increasing UV dose, a plateau level of approximately 40 SCE/cell was reached. This contrasts with the approximately linear increases seen with 8-MOP or 5-MOP over a wider concentration range yielding approximately 60 to 70 SCE/cell. The explanation underlying this plateau in SCE induction by 5-ANG is not known; the reason that the plateau is only 40 SCE/cell, whereas other psoralen compounds can induce over 60 SCE/cell, is also not known. Whether this plateau is characteristic of other angular psoralens remains to be determined.

The pattern of induction of both SCE and gene mutations by 5-ANG also differed from the pattern obtained for 8-MOP, 5-MOP, and 3-CP. The contrast is best illustrated in figure 4. The 3 linear furocoumarins all have nearly identical ratios of induced mutations to induced SCE but the ratio of induced mutations to induced SCE for 5-ANG was much higher. These results imply that the DNA lesions formed by 5-ANG reacting with DNA present a different spectrum than the damage formed by the 3 linear psoralens. Carrano et al. (*37*) had published results that the ratio of induced mutations to induced SCE can vary considerably depending on the type of compound and thus the type of induced DNA damage. It seems likely that the efficiency of induction of each event per DNA lesion was the same for 3-CP, 8-MOP, and 5-MOP, even though higher concentrations of 3-CP, compared with either 8-MOP or 5-MOP, were needed for similar increases in either SCE or mutations to be achieved. This conclusion assumes that the binding of the 3 linear psoralens to DNA of CHO follows the same pattern reported for in vitro binding to purified DNA.

The spectrum of DNA damage induced by these 4 psoralens divides the linear compounds from the angular one and does not distinguish the cross-linking 5-MOP and 8-MOP from the non-cross-linking 5-ANG and 3-CP. One might have expected that the cross-linking compounds would be distinguished from the non-cross-linking ones, inasmuch as a cross-link presents a completely different type of DNA damage compared with monoadducts. The explanation for the similarity in the ratio of induced mutations:induced SCE for 5-MOP, 8-MOP, and 3-CP (which eliminates the differences in binding affinity to DNA) is based on the concentrations of psoralen and the dose of UV light used. Under our experimental conditions, we think it likely that few cross-links were formed by 8-MOP or 5-MOP and the monoadducts formed would be similar to those produced by 3-CP and different from those produced by 5-ANG, the angular psoralen.

Sahar et al. (*38*) examined the relative contribution of monoadducts and cross-links formed by 8-MOP to the induction of SCE in CHO cells using a controlled laser pulse to provide brief pulses of UV irradiation. They found that cross-links were two to three times more efficient than monoadducts at promoting SCE. Most SCE induced by 8-MOP are probably due to monoadducts because the ratio of cross-links to monoadducts is low under most conditions.

We did not measure the number of cross-links/cell, but a useful comparison can be made based on the results of Cech and co-workers (*39*). They measured the production of cross-links by 8-MOP and UV in vitro in isolated calf thymus DNA by examining the DNA in the electron microscope. Under their conditions, 3×10^4 J/m^2 and 93×10^{-6} *M* 8-MOP, they found approximately 1 cross-link/1,000 base pairs. We detected significant increases in SCE using ten to twenty times less psoralen and thirty times less UV. Because Cech et al. also showed that cross-link formation rapidly decreased as the psoralen concentration decreased, the number of cross-links in our experiments was probably too low to contribute significantly to the induction of SCE.

The expectation that angular psoralens might be safer in PUVA therapy is not supported by the observation that 5-ANG induced SCE and mutations. Preliminary experiments that demonstrated an induction of tumors in animals by 5-ANG are consistent with our in vitro findings (Pathak

MA: Personal communication). Whether induction of SCE or mutations is a better indicator of carcinogenicity for psoralens depends on the relative amounts of 5-ANG, 8-MOP, and 5-MOP needed to induce tumors.

On an equimolar basis, 3-CP was much less active than 8-MOP, 5-MOP, or 5-ANG at inducing SCE, mutations, or cell killing. These findings are consistent with the observation that 3-CP is less effective than 8-MOP on an equimolar basis at killing yeast cells or at inducing mutations in the nuclear DNA of yeast (*11*). However, we found that sufficiently high concentrations of 3-CP could induce levels of SCE and mutations comparable to those induced by 8-MOP and 5-MOP. These results can best be interpreted on the basis of weaker binding to DNA by 3-CP compared with 8-MOP, as reported by Isaacs et al. (*35*).

At equal concentrations to 8-MOP, 3-CP did not induce tumors in mice, although 8-MOP induced them in 90% of treated animals (*40, 41*). Our in vitro results suggest that 3-CP could induce tumors if sufficiently high concentrations are used. The observation that 3-CP was effective at clearing psoriasis is promising (*40*), but the results must be viewed with caution, however, because the investigators used twenty times more 3-CP than 8-MOP to achieve equivalent levels of clearing of psoriasis. Studies testing 3-CP for the induction of tumors in animals with higher levels of 3-CP than 8-MOP must be performed before one can conclude that 3-CP poses less risk to humans than 8-MOP.

The mechanism underlying PUVA therapy is not known. Although it may be that several mechanisms of cell killing can be effective in PUVA therapy, we have no evidence at this time to eliminate the hypothesis that inhibition of DNA synthesis is the primary means of inhibiting growth of affected cells. In the PUVA treatments used to date, cross-links may not be involved. Cech et al. (*39*) found less than 1 cross-link in 10^6 base pairs in DNA from guinea pigs treated under conditions similar to PUVA treatments in humans. Treatment regimens have not, however, optimized the formation of cross-links which would be more effective than monoadducts at inhibiting DNA synthesis. The synthesis of compounds which have a lower ratio of induced mutations to induced SCE (and presumably a higher ratio of cross-links to monoadducts) may prove to be effective in PUVA therapy. Similarly, regimens that require initial low doses of UV, followed by a second longer dose of UV after the unreacted psoralen has been eliminated from the body, should increase the level of cross-links in DNA of skin and have already been suggested as a means of modifying PUVA treatment (*42*).

REFERENCES

(*1*) Parrish JA, Fitzpatrick TB, Pathak MA, et al: Photochemotherapy of psoriasis with oral methoxypsoralen and longwave ultraviolet light. N Engl J Med 291:1207–1212, 1974

(*2*) Melski JW, Tanenbaum L, Parrish JA, et al: Photochemotherapy for psoriasis with orally administered methoxsalen. J Invest Dermatol 68:328–335, 1977

(*3*) Fitzpatrick TB, Parrish JA, Pathak MA: Phototherapy of vitiligo. *In* Sunlight and Man: Normal and Abnormal Photobiologic Responses (Fitzpatrick TB, Pathak MA, Harber LC, et al, eds). Tokyo: Univ Tokyo Press, 1974, pp 783–793

(*4*) Stern RA, Thibodeau LA, Kleinerman RA, et al: Risk of cutaneous carcinoma in patients treated with oral methoxsalen photochemotherapy for psoriasis. N Engl J Med 300:809–813, 1979

(*5*) Ashwood-Smith MJ, Poulton GA, Barker M, et al: 5-Methoxypsoralen, an ingredient in several suntan preparations, has lethal, mutagenic, and clastogenic properties. Nature 285:407–409, 1980

(*6*) Bridges BA, Mottershead RP, Knowles A: Mutation induction and killing of *Escherichia coli* by DNA adducts and crosslinks: A photobiological study with 8-methoxypsoralen. Chem Biol Interact 27:221–233, 1979

(*7*) Bridges BA, von Wright A: Influences of mutations at the *rep* gene on survival of *Escherichia coli* following ultraviolet irradiation or 8-methoxypsoralen photosensitization: Evidence for a *recA*$^+$ *rep*$^+$-dependent pathway for repair of DNA crosslinks. Mutat Res 82:229–238, 1981

(*8*) Pani B, Babudri N, Venturini S, et al: Mutation induction and killing of prokaryotic and eukaryotic cells by 8-methoxypsoralen, 4,5′-dimethylangelicin, 5-methylangelicin, 4′-hydroxymethyl, 4,5′-dimethylangelicin. Teratogen, Carcinogen, Mutagen 1:407–415, 1981

(*9*) Averbeck D, Moustacchi E: Genetic effect of 3-carbethoxypsoralen, angelicin, psoralen, and 8-methoxypsoralen plus 365 nm irradiation in *Saccharomyces cerevisiae*. Mutat Res 68:133–148, 1979

(*10*) ———: Decreased photo-induced mutagenicity of monofunctional as opposed to bifunctional furocoumarins in yeast. Photochem Photobiol 31:475–478, 1980

(*11*) Averbeck D, Averbeck S, Dall'Acqua F: Mutagenic activity of three monofunctional and three bifunctional furocoumarins in yeast (*Saccharomyces cerevisiae*). Farmaco [Sci] 36:492–505, 1981

(*12*) Scott BR, Pathak MA, Mohn GR: Molecular and genetic basis of furocoumarin reactions. Mutat Res 39:29–74, 1976

(*13*) Scott BR, Maley MA: Mutagenicity of monoadducts and cross-links induced in *Aspergillus nidulans* by 8-methoxypsoralen plus 365 nm radiation. Photochem Photobiol 34:63–67, 1981

(*14*) Burger PM, Simons JW: Mutagenicity of 8-methoxypsoralen and long-wave ultraviolet irradiation in diploid human skin fibroblasts: An improved risk estimate in photochemotherapy. Mutation Res 63:371–380, 1979

(*15*) ———: Mutagenicity of 8-methoxypsoralen and long-wave ultraviolet irradiation in V79 Chinese hamster cells. A first approach to a risk estimate in photochemotherapy. Mutat Res 60:381–389, 1979

(*16*) Schenley RL, Hsie AW: Interaction of 8-methoxypsoralen and near-UV light causes mutation and cytotoxicity in mammalian cells. Photochem Photobiol 33:179–185, 1981

(*17*) Babudri N, Pani B, Venturini S, et al: Mutation induction and killing of V79 Chinese hamster cells by 8-methoxypsoralen plus near-ultraviolet light: Relative effects of monoadducts and crosslinks. Mutat Res 91:391–394, 1981

(*18*) Latt SA, Juergens LA: Determinants of sister chromatid exchange frequencies in human chromosomes. *In* Population Cytogenetics: Studies in Humans (Hook EB, Porter I, eds). New York: Academic Press, 1977, pp 217–236

(*19*) Latt SA, Allen JW, Stetten G: In vitro and in vivo analyses of chromosome structure, replication, and repair using BrdU-33258 Hoechst techniques. *In* International Cell Biology 1976–1977 (Brinkley BR, Porter KR, eds). New York: Rockefeller Univ Press, 1977, pp 520–527

(*20*) CARTER DM, WOLFF K, SCHNEDL W: 8-Methoxypsoralen and UVA promotes sister chromatid exchanges. J Invest Dermatol 67:548–551, 1976

(*21*) LATT SA, LOVEDAY KS: Characterization of sister chromatid exchange induction by 8-methoxypsoralen plus near UV light. Cytogenet Cell Genet 21:184–200, 1978

(*22*) MOURELATOS D, FAED MJ, JOHNSON BE: Sister chromatid exchanges in human lymphocytes exposed to 8-methoxypsoralen plus long wave UV radiation prior to incorporation of bromodeoxyuridine. Experientia 33:1091–1093, 1977

(*23*) WAKSVIK H, BROGGER A, STENE J: Psoralen/UVA treatment and chromosomes. I. Aberrations and sister chromatid exchange in human lymphocytes in vitro and synergism with caffeine. Hum Genet 38:195–207, 1977

(*24*) SCHULER CF, LATT SA: Sister chromatid exchange induction resulting from systemic, topical and system-topical presentations of carcinogens. Cancer Res 39:2510–2514, 1979

(*25*) AMES BN, DURSTON W, YAMASAKI E, et al: Carcinogens are mutagens: A simple test system combining liver homogenates for activation and bacteria for detection. Proc Natl Acad Sci USA 70:2281–2285, 1973

(*26*) HOLLSTEIN M, MCCANN J, ANGELOSANTO FA, et al: Short-term tests for carcinogens and mutagens. Mutat Res 65:133–226, 1979

(*27*) PERRY P, EVANS HJ: Cytological evidence of mutagen-carcinogen exposure by sister chromatid exchange. Nature 251:256–258, 1974

(*28*) POPESCU NC, TURNBULL D, DIPAOLO JA: Sister chromatid exchange and chromosome aberration analysis with the use of several carcinogens and non-carcinogens. J Natl Cancer Inst 59:289–293, 1977

(*29*) ZAJDELA F, BISAGNI E: 5-Methoxypsoralen, the melanogenic additive in sun-tan preparations, is tumorigenic in mice exposed to 365 nm UV radiation. Carcinogenesis 2:121–127, 1981

(*30*) FRY RJ, LEY RD, GRUBE DD: Photosensitized reactions and carcinogenesis. Natl Cancer Inst Monogr 50:39–43, 1978

(*31*) O'NEILL JP, BRIMER PA, MACHANOFF R, et al: A quantitative assay of mutation induction at the *hypoxanthine-guanine phosphoribosyl transferase* locus in Chinese hamster ovary cells (CHO/HGPRT system): Development and definition of the system. Mutat Res 45:91–101, 1977

(*32*) O'NEILL JP, HSIE AW: Phenotypic expression time of mutagen-induced 6-thioguanine resistance in Chinese hamster ovary cells (CHO/HGPRT system). Mutat Res 59:109–118, 1979

(*33*) PERRY P, WOLFF S: New Giemsa method for the differential staining of sister chromatid exchanges. Nature 251:256–258, 1974

(*34*) LOVEDAY KS, LATT SA: A high buoyant density fraction in mammalian DNA: Characterization and impact on the detection of heteroduplex DNA. Exp Cell Res 136: 177–187, 1981

(*35*) ISAACS ST, WIESEHAHN G, HALLICK LM: In vitro characterization of the reaction of four psoralen derivatives with DNA. Natl Cancer Inst Monogr 66:21–30, 1984

(*36*) AVERBECK D, MOUSTACCHI E, BISAGNI E: Biological effects and repair of damage photoinduced by a derivative of psoralen substituted at the 3,4 reaction site: Photoreactivity and lethal effect in yeast. Biochim Biophys Acta 518:464–481, 1978

(*37*) CARRANO AV, THOMPSON LH, LINDL PA, et al: Sister chromatid exchange as an indicator of mutagenesis. Nature 271:551–553, 1978

(*38*) SAHAR E, KITTREL C, FULGHUM S, et al: Sister chromatid exchange induction in Chinese hamster ovary cells by 8-methoxypsoralen and brief pulses of laser light: Assessment of the relative importance of 8-methoxypsoralen–DNA monoadducts and crosslinks. Mutat Res 83:91–105, 1981

(*39*) CECH T, PATHAK MA, BISWAS RK: An electron microscopic study of the photochemical cross-linking of DNA in guinea pig epidermis by psoralen derivatives. Biochim Biophys Acta 562:342–360, 1979

(*40*) DUBERTRET L, AVERBECK D, ZAJDELA F, et al: Photochemotherapy (PUVA) of psoriasis using 3-carbethoxypsoralen, a non-carcinogenic compound in mice. Br J Dermatol 101:379–389, 1979

(*41*) DUBERTRET L, AVERBECK D, BISAGNI E: Bases expérimentales et premiers essais d'une photochimiothérapie du psoriasis par une furocoumarine monofunctionnelle non cancérigène, le 3-carbethoxypsoralène. C R Acad Sci [D] (Paris) 288:975–979, 1979

(*42*) HANAWALT PC, SU-CHIN L, PARSONS CP: DNA repair responses in human skin cells. J Invest Dermatol 77:86–90, 1981

DISCUSSION

M. Pathak: Dr. Loveday, your observations tell about psoralens increasing the frequency of SCE. I would like to hear Dr. Carter's and Dr. Wolff's views of in vivo conditions that may be operating when you have 8-MOP or 5-MOP inducing some similar increases in SCE.

D. M. Carter: The concentrations that I used with lymphoblastoid cells and with peripheral blood leukocytes are substantially lower, perhaps 100-fold lower, than those reported today. We use 10^{-8} *M* psoralen which produces an effect. The biologic significance of concentrations of this sort is more comparable to a clinical situation than the concentrations reported today. I do not know for certain but have tried to calculate what the cutaneous concentrations might be. I think they are in the 10^{-8} *M* range. I have heard a recurring theme all day. The chemists have worked with elegant controlled conditions; the biologists and the clinicians have less control.

J. E. Hearst: I think the ideal place to pick up a difference between monoaddition and cross-link is low drug concentration followed by a high dose of UV because we know the light dose dependence in the 2 situations is different and we would like to amplify that. I believe that the low drug concentration range is the right place to start if you want to show a difference in cross-linking and monoaddition.

K. Wolff: In the in vivo situation in treated patients there is no increased incidence of SCE, but this was done only with 8-MOP, not with 5-MOP. I do not know whether a study exists with 5-MOP; I doubt it. In the patients we studied, the only group or subgroup who showed an increased incidence of SCE were psoriatics with atrophy, not those treated with PUVA.

SESSION V

Therapeutic, Cytotoxic, and Carcinogenic Aspects of Psoralens and Longwave Radiation Therapy

Safety and Therapeutic Effectiveness of Selected Psoralens in Psoriasis[1]

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ABSTRACT—This review summarizes the most important facts regarding the clinical effectiveness of oral photochemotherapy for psoriasis with UV radiation at 320–400 nm (UVA) and selected furocoumarins. The most widely used compound is 8-methoxypsoralen (8-MOP); its effectiveness has been documented by many clinical trials. Oral 5-methoxypsoralen (5-MOP) has been evaluated as an alternative drug because it is less erythemogenic and thus reduces the danger of accidental overexposure. It has been as effective as 8-MOP in clearing psoriasis, but the UVA doses required were considerably higher. Although oral 4,5′,8-trimethylpsoralen does not clear psoriasis satisfactorily, excellent treatment results have been recorded when it was applied topically. The 3 drugs produce bifunctional adducts with DNA (cross-links), which may be of particular importance for mutagenesis and tumor formation. In an attempt to reduce possible oncogenic hazards, investigators are currently testing non-cross-linking furocoumarins for their therapeutic effectiveness. At present, these compounds are available for topical use only. 3-Carbethoxypsoralen has been reported to produce excellent treatment results by others but was ineffective in our clinical trials. Similarly disappointing was the application of 4,5′-dimethylangelicin and 5-methylangelicin. Although monofunctional compounds also inhibit cell proliferation in vitro, it appears that cross-linking is a prerequisite for the therapeutic success in psoriasis. The actual importance of cross-links, however, remains to be clarified. If one considers the beneficial effects of photochemotherapy in respect to the potential risk and safety, PUVA, at the present stage, represents the treatment of choice in severe widespread psoriasis. — Natl Cancer Inst Monogr 66: 159–164, 1984.

Photochemotherapy is defined as a treatment that depends on the interaction of nonionizing electronmagnetic radiation and a photosensitizing compound within the tissue that results in a therapeutically desired beneficial effect. Photochemotherapy can be administered orally in the form of a photosensitizing furocumarin followed by whole-body longwave UVA radiation or the photosensitizer can be applied topically. Of the psoralens, 8-MOP is the most widely used compound (*1*), but 5-MOP (*2*) and TMP have also been tested clinically. Pilot studies have been performed with some derivatives of angelicin, an angular furocoumarin (*3, 4*), and a new synthetic compound known as 3-CP (*5*). The rationale of clinicians using PUVA therapy is to bring psoriasis into remission by repeated, controlled photosensitization reactions (which are monitored to remain within a therapeutically desired range) and to subject the patient, after clinical suppression of disease activity, to maintenance treatment to prevent recurrences. Because photoactivation of the psoralen within the tissue can take place only in tissue compartments that are reached and penetrated by UVA, the targets of the photochemical reactions in PUVA are the cells of the superficial layers of the skin. Psoriasis is a disease which involves this skin compartment, and oral PUVA therapy therefore represents an example of selective chemotherapy of the target tissue that does not affect internal organs. However, circulating blood cells which percolate through the dermal capillaries may be affected by PUVA, and the cellular immune system of the skin interacts with the immune system of the whole body (*1*). It is in this respect that changes induced by PUVA in the skin may have systemic consequences.

The dramatic effectiveness of oral 8-MOP plus UVA in psoriasis has been documented widely by studies both in individual research centers (*1*) and 3 cooperative clinical trials in the United States and Europe (*6–8*). Recognized as the most efficient treatment available for psoriasis, PUVA also offers the advantages of ambulatory treatment, continuous suppression of disease activity by maintenance therapy, and high patient acceptance. Acute side effects are low if guidelines for appropriate dosimetry are observed. Because psoralens interact with DNA under the influence of UV light and experimental evidence suggests that these events are not only mutagenic in bacteria but can also produce tumors in experimental animals, concerns have been expressed as to the long-term side effects and safety of PUVA treatment. Therefore, it is the aim of our review to summarize the most important facts concerning the clinical effectiveness of PUVA therapy with 8-MOP and other psoralens[3] and to discuss these beneficial effects in respect to the potential risk and safety of this treatment.

CLINICAL EFFECTIVENESS

8-Methoxypsoralen Plus Irradiation

Practically all forms of psoriasis respond to PUVA therapy; this includes eruptive and chronic plaque psoriasis; unstable, exudative, and pustular forms; erythroderma; and psoriasis of palms and soles (*1*). Lesions respond first by flattening and by a decrease of scaling and erythema. As pigmentation (also induced by this treatment) increases, the lesions disappear completely and a uniform, esthetically pleasing tan develops. A better than 90% clearing rate was reported for the original 21 and 150 patients (*9, 10*), and

ABBREVIATIONS: UVA = UV radiation at 320–400 nm; 8-MOP = 8-methoxypsoralen; 5-MOP = 5-methoxypsoralen; TMP = 4,5′,8-trimethylpsoralen; 3-CP = 3-carbethoxypsoralen; PUVA = psoralen plus UVA; J = joules.

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[3] We will focus on systemic (oral) photochemotherapy and will refer to topical treatment only when data on systemic PUVA are not available.

this was confirmed by subsequent reports from other centers (*1*). In 572 patients continuously treated by our group, a clearing rate of 93% was achieved with a mean of 15 individual treatments given over a period of 30 days and requiring a total cumulative UVA dose of 79 J/cm^2 (*11*). Similar results were obtained in the 2 cooperative multicenter trials (*6, 7*) conducted in the United States and the multicenter trial conducted in Europe (*8*), totaling approximately 5,000 patients. Of the 1,139 patients reported by Melski et al. (*6*), 88% of the patients showed complete clearing, 69% of whom cleared with less than 30 treatments, whereas 19% required 30 or more treatment sessions; 9% had dropped out of treatment, and 3% were considered failures. In this study, the mean total UVA requirement for clearing ranged from 191 J/cm^2 for skin type I to 296 J/cm^2 for type IV patients.[4] In the European multicenter study (*8*) that comprised 3,175 patients, complete clearing was also achieved in 89% of the patients, but only 19 exposures (mean) and 103 J/cm^2 (mean) were required to achieve this result in all skin types. This represents one-third to one-half the dose given to patients treated according to the United States protocol. The reasons for this rest in the differences in treatment protocols used; these have been discussed at length elsewhere (*1*).

Psoriatic erythroderma is also amenable to PUVA therapy, even though a higher number of treatments and more time are required to induce remissions and eventual failure rates are higher, ranging from 14 to 16%, than they are in other forms of psoriasis when they range from 2.6 to 6.5% (*6, 8*). By contrast, dramatic results have been obtained in pustular psoriasis of the palms and soles and in pustular psoriasis of von Zumbusch (*6, 12*), a severe inflammatory form of psoriasis with leukocytosis and fever. In 1 series, 100% of patients cleared, and, concomitant with the cutaneous improvement, systemic symptoms abated and leukocytosis reverted to normal (*12*).

Of particular importance is the fact that PUVA controls psoriatic disease in patients who, before the PUVA era, had to be maintained on cytotoxic agents or systemic corticosteroids for control of their disease (*13*). Withdrawal of previous therapy is often followed by considerable flares, but clearing can eventually be achieved even though more time is required for a resolution of lesions than in patients not previously treated with these agents. Finally, PUVA exerts a beneficial effect on psoriatic arthropathy in nonspondylitic patients in whom a 49% improvement of articular index has been described (*14*); however, evidence that it may prevent destructive joint disease is still forthcoming.

One of the advantages of PUVA is that, as maintenance treatment, it controls recurrences and permits patients to be kept in remission for prolonged periods. With the original maintenance treatment regimen of 1 to 2 treatments per week that are gradually tapered to 1 every 3 or 4 weeks, 80% of the patients were kept in remission (*9, 10*); in the series of Melski et al. (*6*), 58% of 1,000 patients were still clear at the time of the examination 1 year after treatment began. Surprisingly, in the European cooperative study, calculations based on life-table techniques have shown that the chances of a patient remaining in remission within a period of 80 weeks are almost the same whether PUVA maintenance treatment is administered or not (*8*). An argument has arisen over the validity of the statistical approach in this study and, indeed, studies by others have shown definitely that recurrence rates are considerably higher with no maintenance therapy than with it (*11, 15*). Because most recurrences appear during the first 2 months after the clearing phase is terminated, a maintenance therapy regimen has been adopted in which patients are kept on infrequent treatments for 2 months, after which no PUVA treatment is given to those still in remission (*1, 11*).

TREATMENT WITH OTHER PSORALENS PLUS IRRADIATION

5-Methoxypsoralen

We evaluated 5-MOP as an alternative drug in PUVA treatment of psoriasis because, on a milligram/kilogram basis, it has a lower photosensitizing and thus less erythemogenic effect on skin (*2*). Determinations of 5-MOP tissue levels in guinea pigs have suggested that this may be the result of lower bioavailability of 5-MOP in the epidermis (*16*). The danger of accidental overexposure should therefore be less, and the incidence of immediate side effects should be lower with this analog. Used in low- (0.6 to 0.8 mg/kg) and high-dose (1.2 to 1.6 mg/kg) regimens, oral 5-MOP has been as effective as 8-MOP in PUVA treatment of psoriasis (*2, 17*), but UVA doses required for clearing are higher [mean total dose = 105 J/cm^2 as compared with 8-MOP (79 J/cm^2)]. A clinically visible phototoxic reaction manifesting as erythema is not required for the involution of psoriatic lesions, and acute side effects due to erythema are avoided. Also, 5-MOP produces less nausea than does 8-MOP but leads to pronounced hyperpigmentation (*2*). A combination of these 2 psoralens in PUVA exposures does not improve therapeutic results, probably because the UVA energy requirements for phototoxic reactions to occur are too different for the 2 drugs (*2*).

4,5′,8-Trimethylpsoralen

Oral TMP, a time-honored drug for repigmentation in patients with vitiligo, has also proved effective in PUVA therapy of psoriasis, but we found the resolution of lesions has been slow, and this may be the reason why controlled clinical trials have not been performed. Administered orally, TMP is less erythemogenic than 8-MOP but leads to pronounced hyperpigmentation. A favorable clinical response has been seen in 9 of 11 patients treated with 40 mg oral TMP and subsequent exposure to sunlight (*18*), but complete clearing was observed only in 3. By contrast, excellent results have been recorded when TMP was used topically, either as a bath or alcoholic solution painted on the skin followed by exposure to UVA (*19*).

[4] Inasmuch as the biologically relevant phototoxic reactions within the skin cannot be measured, the UVA doses delivered to induce these reactions are recorded as indirect parameters for phototoxic doses used.

3-Carbethoxypsoralen

Attempts to reduce oncogenic hazards of PUVA led to the introduction of non-cross-linking psoralens into PUVA therapy. A monofunctional synthetic psoralen with remarkable photoactivity, 3-CP has been reported to be neither erythemogenic nor oncogenic in mice and to show a favorable effect on psoriatic lesions in man after topical application and subsequent irradiation with UVA (*5*). Of 10 patients treated, excellent results were described in 4 and a medium response in 3 who required from 8 to 48 treatments and a total cumulative UVA dose of 120 to 720 J/cm^2 (*5*). Our results have been disappointing because an effect better than the control (UVA alone) was observed in only 1 of 10 patients within a 3-week treatment period (unpublished observation).

Derivatives of Methylangelicin

Various derivatives of angelicin have been developed that, as angular furocumarins, produce only monofunctional adducts with DNA. 4,5′-Dimethylangelicin and 5-methylangelicin appeared as promising compounds because of their high monofunctional photoreactivity with DNA, their capacity to inhibit cell division, and the fact that they are nonerythemogenic when applied to guinea pig skin (*3, 4*). However, pilot studies performed in our laboratory revealed poor clinical results when the agents were applied topically to psoriatic lesions and exposed to UVA 1 hour later (unpublished observations).

SAFETY AND RISKS OF TREATMENT

Short-term side effects are mainly due to overdosage, and because they present as clinically defined symptoms of skin phototoxicity, they are predictable. They consist of pruritus, erythema, edema, or blistering and usually can be avoided if the guidelines for dosimetry are rigidly observed (*1, 9, 10*). A comparison of patients treated by us (*1*) in 1974, when the criteria for dosimetry were still being developed with patients who received PUVA therapy, shows a drastic reduction of side effects in the latter patients (pruritus: 35% vs. 21%; localized +++ erythema: 20% vs. 6%; blistering: 10% vs. 2%). Nausea, which is due to intolerance of 8-MOP, occurs frequently (13%) but is rarely severe enough to cause cessation of treatment; some rare and inconsequential side effects have been reviewed elsewhere (*1*). The 3 multicenter trials (*6–8*) confirmed the low incidence of side effects due to overdosage; it is in this respect that PUVA treatment can be considered safe.[5]

Long-term safety is a different issue. With regard to the possible effects of psoralens on internal organs, it is now established that these compounds are not hepatotoxic, nor do they induce side effects in other organ systems. Significant abnormal laboratory data have not been observed in the 5,000 patients of the 3 multicenter trials (*6–8*), and liver biopsies performed before and after PUVA treatment failed to reveal any change (*20*). However, concerns have been expressed as to the long-term effects of PUVA on the eye, on circulating blood cells (which can be affected by psoralen–UVA reactions as they circulate through dermal capillaries), the immune system, and the skin, in which repeated phototoxic injuries can result in cumulative actinic degeneration and the induction or promotion, or both, of skin cancer (*1*).

Most of these concerns have already been discussed at length by others at this Conference and need not be reiterated here. Data presented on the potential risks of PUVA make it appear premature for us to speculate on the long-term safety of this treatment, but its extraordinary effectiveness and the lack of alternate options for therapy, which are innocuous but equally effective, justify a more differentiated look at this problem. The clinical situation requires that risk-to-benefit ratio decisions weigh potential long-term side effects against the severity of the disease to be treated and reduce potential or established risks to a minimum. Let us return to the area of greatest concern, i.e., the question of carcinogenesis in psoriatics treated with PUVA. Several risk factors are now recognized which include skin type (and thus sensitivity to the effect of radiant energy), previous treatments (which are carcinogenic), and the total cumulative phototoxic dose delivered to the patient. Therefore, a reduction of the cumulative dosage should increase the safety of PUVA, and data available to date indicate that this can be achieved by 1) treatment regimens devised for rapid clearing, such as the European protocol; 2) arbitrary, individualized maintenance treatments adjusted to the patient's needs that avoid unnecessary exposures, and 3) combination of PUVA with other forms of treatment. As in polychemotherapy of cancer, the goal of such combinations is the potentiation of beneficial effects and the reduction of the adverse type of the individual components. Topical corticosteroids, anthralin or tar, and systemic methotrexate have been combined with PUVA, but the greatest advance has been made with the adjuvant, systemic administration of an aromatic retinoid (*20*). This combination, also termed, "chemophotochemotherapy" (*1*), accelerates the response of psoriasis to PUVA and thus reduces not only the duration of treatment but also the total cumulative dose of UVA by 40 to 50% (*21*). As patients enter maintenance therapy with low energy requirements per exposure, they can be kept under control by low-dose maintenance PUVA treatment, which results in an additional reduction of the UVA radiation load applied over prolonged periods.

Evidence that the approach of reducing long-term side effects by a reduction of the cumulative phototoxic dose may be correct is based on the observations that 1) the incidence of tumors in patients treated according to the European protocol is smaller than in the United States cooperative trial (*22*), and 2) we have seen no tumors in patients who we treated with aromatic retinoids and PUVA and followed for 4 years. Conversely, no unequivocal evidence exists that this apparently safer approach is indeed a function of a lower, biologically relevant phototoxic dose which is not measured but extrapolated from the UVA doses delivered to the patient. In patients treated with PUVA, Lerche and co-workers (*23*) and Cech et al. (*24*)

[5] This statement is valid only if treatment is performed properly according to established criteria; a generalized phototoxic reaction with overall erythema, edema, and blistering may be a life-threatening condition.

detected cross-links in epidermal DNA only when psoralens had been administered in high doses, but not with doses usually used in clinical treatment. Because DNA cross-links are widely considered essential molecular lesions of PUVA treatment, the question arises how important cross-link formation is for a clinically beneficial result in psoriasis. Three psoralens known to be effective clinically, 8-MOP, 5-MOP, and TMP, are also strong cross-linking agents, so that this activity and clinical effectiveness appear to correlate. On the other hand, monofunctional, non-cross-linking compounds, such as 3-CP and angelicin derivatives, have also been considered potentially effective because they inhibit cellular proliferation. Clinically, these agents have shown only low or no effectiveness in psoriasis. Pohl and Christophers (*25*) demonstrated in cultured fibroblasts that, after UVA plus 8-MOP in vitro and subsequent removal of unbound 8-MOP from the medium, additional UVA irradiation resulted in further inhibition of DNA synthesis, which they interpreted to indicate that a conversion of monoadducts to cross-links had been induced by the second UVA dose. Hanawalt (*26*) proposed that a second UVA dose given in PUVA treatment after nonbound 8-MOP was cleared from the tissue could minimize oncogenic risks by maximizing cross-links at the expense of monoadducts and at the same time enhance the therapeutic effect of PUVA. Preliminary experiments in our laboratory with this split-dose PUVA regimen have been disappointing, as psoriasis thus treated showed no better response than with regular PUVA therapy.

Monofunctional psoralens are mutagenic as are bifunctional psoralens; whether few but large phototoxic doses are more harmful than many small doses and whether an aggressive regimen with rest periods is safer or carries more risks than an approach with continuous nonaggressive treatment are unresolved questions. One can make a case for any of these possibilities by comparing the various regimens in use and the incidence of tumors observed in PUVA patients both in the United States (*27, 28*) and Europe (*29, 30*), but too many variables are involved to permit meaningful conclusions. The phenomenon of focal epidermal dystrophy is a good illustration of this point. Cox and Abel (*31*) observed focal epidermal dystrophy in almost 50% of their patients treated for up to 1 year, whereas no such changes were seen by us in 243 patients treated up to 4 years (*32*). In the study of Cox and Abel (*31*), clearing periods were described as having been "usually less than six months" and thus were extremely long compared with the less than 1-month clearing period of our study. One of several possible interpretations of this discrepancy was that their patients had been subjected to long periods of almost continuous PUVA therapy, with few rest periods available for the epidermis to recover from PUVA-induced injury (*1*). Conversely, in patients with "PUVA-induced mottling," a chronic poikilodermatous skin condition due to repeated phototoxic exposures of overdosed skin, our group (*32*) detected epidermal dystrophy in 45% of patients, in whom it could be interpreted as the result of single, large doses of PUVA. Finally, we are now seeing focal epidermal dystrophy in some long-term PUVA patients with high cumulative doses of UVA and here the phenomenon may reflect an excessive UVA radiation load accumulated over prolonged periods (*33*).

The mode of action of PUVA in psoriasis is unknown but because it induces monoadducts and cross-links, both of which interfere with replication of DNA and thus cellular proliferation, it is a popular belief that these molecular lesions are the decisive events in the control of epidermal hyperproliferation in this disease. However, monofunctional psoralens which induce only monoadducts but do inhibit cell proliferation are virtually ineffective in the treatment of psoriasis and, as they are also mutagenic and carcinogenic, they are certainly not safer than the clinically highly effective cross-linking psoralens 8-MOP and 5-MOP. Nevertheless, cross-links have not been detected in psoriatic skin treated with PUVA doses that induce remissions, and the split-dose PUVA regimen, which allegedly converts monoadducts into cross-links and thus increases their number, is clinically no better than PUVA alone. Again, the question arises as to how important both cross-link formation and (in more general terms) psoralen–DNA interactions are in PUVA therapy of psoriasis. Psoralens and UVA interact not only with DNA, but they also interfere with RNA and protein synthesis. We know from clinical and laboratory experiments that PUVA abrogates or modulates immunologic functions (*1*). As a clinical treatment, PUVA is effective not only in psoriasis but also in mycosis fungoides and other cutaneous lymphomas and, more importantly, in various lymphocytic disorders or in cutaneous mastocytosis that are not hyperproliferative in nature (*34*). Moreover, DNA lesions induced by PUVA do occur but they may not be required for a beneficial effect of this treatment in psoriasis. A search for the modes of action of psoralens and UVA in psoriasis may reveal mechanisms not dependent on DNA lesions; we believe that such investigations will eventually be better suited to solve the problem of long-term safety of PUVA.

For the present, as clinicians, we have to recognize that PUVA is the most effective treatment for severe psoriasis, but at least theoretically, some potential long-term risks cannot be denied. Risk-to-benefit ratio decisions have to be made, but we should recognize that few other options for treating severe, generalized psoriasis exist. Currently, PUVA centers have been established in all parts of the world, and government approval has been and is being granted in an increasing number of countries. With the ongoing multicenter trials in the United States and in Europe, in which an extraordinarily large number of patients is being followed continuously, PUVA is the best controlled and monitored therapeutic principle ever introduced into dermatology (*35*).

Severe, widespread psoriasis is a devastating disease, but this also applies to localized psoriasis on exposed parts of the body or the palms where the disease can lead to gross disfigurement and result in disruption of patients' professional, social, and private lives. Nonetheless, in view of the potential risks of PUVA, this treatment should be limited to those patients with severe psoriasis who can be properly monitored and controlled by clinicians thoroughly familiar with this treatment.

REFERENCES

(1) WOLFF K, HÖNIGSMANN H: Clinical aspects of photochemotherapy. Pharmacol Ther 12:381–418, 1981

(2) HÖNIGSMANN H, JASCHKE E, GSCHNAIT F, et al: 5-Methoxypsoralen (bergapten) in photochemotherapy of psoriasis. Br J Dermatol 101:369–378, 1979

(3) DALL'ACQUA F, VEDALDI D, CAFFIERI S, et al: New monofunctional reagents for DNA as possible agents for the photochemotherapy of psoriasis: Derivatives of 4,5′-dimethylangelicin. J Med Chem 24:178–184, 1981

(4) DALL'ACQUA F, VEDALDI D, GUIOTTO A, et al: Methylangelicins: New potential agents for the photochemotherapy of psoriasis. Structure–activity study on the dark and photochemical interactions with DNA. J Med Chem 24:806–811, 1981

(5) DUBERTRET L, AVERBECK D, ZAJDELA F, et al: Photochemotherapy using 3-carbethoxypsoralen, a non-carcinogenic compound in mice. Br J Dermatol 101:379–389, 1979

(6) MELSKI JW, TANENBAUM L, PARRISH JA, et al: Oral methoxsalen photochemotherapy for the treatment of psoriasis: A cooperative clinical trial. J Invest Dermatol 68: 328–335, 1977

(7) Clinical cooperative study of PUVA-48 and PUVA-64: Photochemotherapy of psoriasis. Arch Dermatol 115: 576–579, 1979

(8) HENSELER T, WOLFF K, HÖNIGSMANN H, et al: Oral 8-methoxypsoralen photochemotherapy of psoriasis. The European PUVA Study: A cooperative study among 18 European centres. Lancet 1:853–857, 1981

(9) PARRISH JA, FITZPATRICK TB, TANENBAUM L, et al: Photochemotherapy of psoriasis with oral methoxsalen and longwave ultraviolet light. N Engl J Med 291:1207–1211, 1974

(10) WOLFF K, HÖNIGSMANN H, GSCHNAIT F, et al: Photochemotherapie bei Psoriasis. Klinische Erfahrungen bei 152 Patienten. Dtsch Med Wochenschr 100:2471–2477, 1975

(11) WOLFF K: PUVA 1979. Klinik und Praxis. *In* Fortschritte der praktischen Dermatologie und Venerologie (Braun-Falco O, Wolff HH, eds), vol 9. Berlin, Heidelberg: Springer-Verlag, 1979, pp 129–138

(12) HÖNIGSMANN H, GSCHNAIT F, KONRAD K, et al: Photochemotherapy for pustular psoriasis (von Zumbusch). Br J Dermatol 97:119–126, 1977

(13) GSCHNAIT F, KONRAD K, HÖNIGSMANN H: Photochemotherapie bei Kortikosteroid-und Methotrexat-behandelten Psoriatikern. Hautarzt 28:632–637, 1977

(14) PERLMAN SG, GERBER LH, ROBERTS RM, et al: Photochemotherapy and psoriatic arthritis. A prospective study. Ann Intern Med 91:717–722, 1979

(15) VELLA BRIFFA D, GREAVES MW, WARIN AP, et al: The influence of maintenance photochemotherapy on the relapse of plaque psoriasis. Br J Dermatol 103(Suppl 18):14–15, 1980

(16) KORNHAUSER A, WAMER WG, GILES AL JR: Psoralen phototoxicity: Correlation with serum and epidermal 8-methoxypsoralen and 5-methoxypsoralen in the guinea pig. Science 217:733–735, 1982

(17) GRUPPER C, BERRETTI B: 5-MOP in PUVA and Re-PUVA—250 patients with a follow-up of three years. *In* Psoriasis (Farber EM, Cox AJ, eds). New York: Grune & Stratton, 1982, pp 503–508

(18) SEHGAL VN, REGE VL, KHARANGATE VN, et al: Photochemotherapy of psoriasis with 4,5′, 8-trimethylpsoralen. Dermatologica 150:316–319, 1975

(19) FISCHER T, ALSINS J: Treatment of psoriasis with trioxsalen baths and dysprosium lamps. Acta Derm Venereol (Stockh) 56:383–390, 1976

(20) ZACHARIAE H, KRAGBALLE K, SØGAARD H: Liver biopsy in PUVA-treated patients. Acta Derm Venereol (Stockh) 59:268–270, 1979

(21) FRITSCH PO, HÖNIGSMANN H, JASCHKE E, et al: Augmentation of oral methoxsalen-photochemotherapy with an oral retinoic acid derivative. J Invest Dermatol 70: 178–182, 1978

(22) HALPRIN KM, COMERFORD M, TAYLOR JR: Skin cancer in patients treated with 8-methoxypsoralen plus longwave ultraviolet radiation. Natl Cancer Inst Monogr 66:185–189, 1984

(23) LERCHE D, SØNDERGAARD J, WADSKOW S, et al: DNA interstrand cross-links visualized by electron microscopy in PUVA-treated psoriasis. Acta Derm Venereol (Stockh) 59:15–20, 1979

(24) CECH T, PATHAK MA, BISWAS RK: An electron microscopic study of the photochemical cross-linking of DNA in guinea pig epidermis by psoralen derivatives. Biochim Biophys Acta 562:342–360, 1979

(25) POHL J, CHRISTOPHERS E: Photoinactivation of skin fibroblasts by fractionated treatment with 8-methoxypsoralen and UV-A. J Invest Dermatol 73:176–179, 1979

(26) HANAWALT PC, CALVIN NM, LIU S-C: DNA damage and carcinogenesis: Split-dose PUVA to minimize risk in photochemotherapy for psoriasis. *In* Psoriasis (Farber EM, Cox AJ, eds). New York: Grune & Stratton, 1982, pp 163–171

(27) STERN RS, THIBODEAU LA, KLEINERMAN RA, et al: Risk of cutaneous carcinoma in patients treated with oral methoxsalen photochemotherapy for psoriasis. N Engl J Med 300:809–813, 1979

(28) ROENIGK HH JR, CARO WA: Skin cancer in the PUVA-48 cooperative study. J Am Acad Dermatol 4:319–324, 1981

(29) HÖNIGSMANN H, WOLFF K, GSCHNAIT F, et al: Keratoses and non-melanoma skin tumors in long-term photochemotherapy (PUVA). J Am Acad Dermatol 3:406–414, 1980

(30) LASSUS A, REUNALA T, IDÄNPÄÄ-HEIKKILÄ J, et al: PUVA treatment and skin cancer. A follow-up study. Acta Derma Venereol (Stockh) 61:141–145, 1981

(31) COX AJ, ABEL EA: Epidermal dystrophy. Occurrence after psoriasis therapy with psoralen and long-wave ultraviolet light. Arch Dermatol 115:567–570, 1979

(32) GSCHNAIT F, WOLFF K, HÖNIGSMANN H, et al: Long-term photochemotherapy: Histopathologic and immunofluorescence observations in 243 patients. Br J Dermatol 103:11–22, 1980

(33) BRENNER W, KONRAD K, HÖNIGSMANN H, et al: Epidermal and dermal changes in long-term PUVA-treated skin. Presented at the International Congress on Photobiology, Strasbourg, July 20–25, 1980

(34) HÖNIGSMANN H: Photochemotherapy with furocoumarins (psoralens). *In* Trends in Photobiology (Hélène C, Charlier M, Montenay-Garestier TH, eds). New York: Plenum Press, 1981, pp 309–320

(35) PARRISH JA, STERN RS, FITZPATRICK TB: Evaluation of PUVA—1980. Its basic nature and toxicity. *In* Dermatology Update, 1982 (Moschella SL, ed). New York: Elsevier, 1982, pp 313–337

DISCUSSION

L. Grossweiner: I would like to suggest as a "take-home" message that because we have no definitive proof that either monoadducts or cross-links are more mutagenic, this should be considered an open question. It would be imprudent to design clinical procedures on the assumption that either monoadducts or cross-links are the more mutagenic lesions.

With 8-MOP at the molecular level, one is faced with a high concentration of highly repairable monoadducts and with a low number of (error-prone) repairable cross-links. The repair mechanisms are different for the 2 types of lesions, and there is no clean experimental procedure for making the distinction between "more mutagenic or less mutagenic" in this situation.

I think that clinicians should not draw conclusions from mutagenicity in microorganisms or in mammalian cell cultures based on the available evidence that either of the 2 types of lesions would be a safer one from the viewpoint of long-term clinical effects.

F. Dall'Acqua: I should like to stress one thing about methylangelicins. Many people say that methylangelicins are much more mutagenic than are psoralens; this is not true. We have done many experiments. Sometimes, especially with repairless strains, some angelicins are more mutagenic than are linear furocoumarins (psoralens). Generally and especially with wild-type strains, we found that angelicins are less mutagenic than are the linear furocoumarins.

Unidentified Participant: I should like to know the point of view of the Boston group on the therapeutic effectiveness of methylangelicins. Dr. Wolff found that 5-methylangelicin is completely inactive, whereas Dr. Pathak previously mentioned that this angelicin shows a good activity. What is the reason of this discrepancy?

In the overview presented by Dr. Wolff, he suggested that new mechanisms other than those involving the photo damage to DNA, i.e., the damage to proteins and the singlet oxygen formation, should be involved. Professor Wolff is inquiring about the real role of the DNA photodamage.

I believe that until now we observed a good correlation. Also, from the therapeutic point of view, between the extent of lesions that furocoumarins induce in DNA in vitro and the effectiveness in vivo both in antiproliferative and therapeutic activity, the correlation has been good.

So I believe that, for methylangelicins at least, the antiproliferative activity is related to their capacity to photoinduce the DNA lesions. Finally, concerning the interesting remarks of Dr. Grossweiner, in distinguishing the carcinogeneic and the mutagenic activity of monofunctional and bifunctional furocoumarins, I should like to stress that we determined that the bifunctional furocoumarins induce about 90% of the monofunctional lesions and only 10% of the bifunctional ones. Therefore, we cannot ignore the great extent of monofunctional lesions that are present and ascribe the biologic consequences (e.g., the mutagenic activity) only to the bifunctional lesions.

M. A. Pathak: I think that, as far as the monofunctional psoralens and their therapeutic effectiveness are concerned, I cannot comment on the success in Boston and the failures in Dr. Wolff's laboratory. The fact is that we did find clearance in localized patches with 5-methylangelicin and less with 4,5′-dimethylangelicin. Other psoralens were totally inactive. Perhaps we have to do more studies along this line before we can say the treatment is ineffective. We have treated 6 patients with 5-methylangelicin: We had good success with 4 and failed with 2.

T. Fitzpatrick: Dr. Averbeck mentioned to me that 3-CP has been shown to be effective by Mizuno in Japan. Whenever you find someone who can do something and make it work and then somebody else cannot repeat it, I tend to believe the person who makes it work. If it works once, it should be reproducible.

Wolff: I am perfectly happy to accept that, although it does not necessarily exclude that the first person was wrong. Of course, our experience is limited, and perhaps we did something wrong or something happened to the compound. Incidentally, Dr. Mizuno has informed me that he has not found 3-CP to be effective. In response to Dr. Grossweiner, I would like to add that, as clinicians, we are caught between what we can do and achieve clinically and the warning voices of the chemists specializing in work with DNA who tell us that a compound can react in certain ways with DNA and that "this and that" may evolve from such interaction. Somehow, we have to deal with this. This response leads to attempts to work with or use psoralens that theoretically may not seem to be as "dangerous" as some others, but 1) no ideal substance exists, and 2) we really do not know what molecular lesions are required for effectiveness in clinical medicine in man. That is the problem.

Safety and Therapeutic Effectiveness of 8-Methoxypsoralen, 4,5′,8-Trimethylpsoralen, and Psoralen in Vitiligo[1, 2]

Madhu A. Pathak, David B. Mosher, *and* Thomas B. Fitzpatrick[3, 4]

ABSTRACT—In this paper, we report on 366 East Indian patients with vitiligo who were treated for 2 to 3 years with either 8-methoxypsoralen (8-MOP), 4,5′,8-trimethylpsoralen (TMP), or psoralen and sunlight. These patients with amelanotic macules had 10 to 70% skin involvement of 1 to 50 years duration. Male and female patients from ages 12 to 70 years were randomly assigned to 8 treatment groups; the study was conducted on a double-blind protocol. Patients in prone and supine positions were exposed to the sun for 45–60 minutes in gradually increasing doses between 11 a.m. and 2 p.m. thrice weekly and 2 hours after oral ingestion of the drug. The various drug dosage schedules investigated included 9 groups: 0.3 and 0.6 mg 8-MOP/kg; 0.8, 1.8, and 3.6 mg TMP/kg; a combination of 0.3 mg 8-MOP and 0.6 mg TMP/kg; 0.6 and 1.2 mg psoralen/kg, and a placebo. For ethical reasons, the placebo group was terminated after 9 to 12 months of therapy. All patients were photographed before enrollment and at intervals of 6, 12, 18, and 26 months during therapy. Of these patients treated for nearly 2 years, the faces of those 45% receiving the combination dose of 8-MOP plus TMP or low-dose 8-MOP (0.3 mg/kg) were fully repigmented, and nearly 60% achieved 75 to 100% repigmentation of the head and neck. The chest, abdomen, and back were repigmented nearly as well and better than the arms and legs. The patients receiving high-dose schedules of TMP and psoralen achieved better repigmentation response than those on lower dosage but still not as good as patients on 8-MOP or the combination group of 8-MOP plus TMP. The patients receiving a low dose 8-MOP for 2 years or more did better than those receiving a high dose. The lips, hands (knuckles and finger tips), feet (ankles and toes), palms, and soles repigmented poorly. Seventy-five percent or more repigmentation of all vitiligo macules occurred in 38% of the patients in the 8-MOP plus TMP group, 31% in the group receiving 0.3 mg 8-MOP/kg, and less than 17% in other groups. The placebo group showed dismal repigmentation. Long-term therapy (between 100 and 300 treatments) is required for successful repigmentation. Despite some chronic phototoxicity changes in vitiliginous macules in patients receiving high-dose 8-MOP, TMP, and psoralen and complaints of nausea and pruritis, the patients tolerated psoralen and 320- to 400-UV radiation therapy fairly well. The photochemotherapy of vitiligo with oral 8-MOP or TMP can be an effective treatment given patient compliance and long-term follow-up. For pigmented (dark-skinned) individuals, 8-MOP is recommended, whereas TMP is recommended for fair-skinned individuals. — Natl Cancer Inst Monogr 66: 165–173, 1984.

The herbal use of psoralens (furocoumarins) for the treatment of leukoderma (vitiligo) in India, Egypt, and the Far Eastern countries dates back to the time before the birth of Christ (*1*).

One can trace psoralen therapy for vitiligo to its roots as far back as 1500 B.C. in early Ayurvedic therapy practiced by ancient Hindu priests. Extracts of Bavachee seeds or *Psoralea corylifolia* and other plants containing psoralens were used in folk remedies of various cultures. Even in the late twentieth century, this form of therapy is being used in India, Pakistan, China, and the Far East. Such plants are known to contain melanin-stimulating furocoumarins, including psoralen, 8-MOP, isopsoralen, 8-isoamylenoxypsoralen, 5-MOP, and 5,8-dimethoxypsoralen (*1, 2*). Similarly, the ancient herbal use of *Ammi majus* Linnaeus, an umbelliferous plant containing certain photoreactive psoralens (e.g., ammoidin or 8-MOP, ammidin or 8-isoamelenoxypsoralen, majudin, or 5-MOP) has been carefully documented by the Egyptians (*1, 3, 4*).

The modern period of psoralen research began in 1938 when Kuske (*5*) investigated phytophotodermatitis of the skin which came in contact with certain psoralen-containing plants and UV light. Artificial light sources such as the Kromayer lamp were found to stimulate melanogenesis. By 1947, Fahmy and Abu-Shady (*3*) had isolated from the powdered seeds of *Ammi majus* L. 3 crystalline psoralens: 8-MOP, 5-MOP, and 8-isoamylenoxypsoralen. In the same era, successful repigmentation was recorded earlier by Uhlmann (*6*) and later by El Mofty (*4, 7*).

Subsequently, several investigators (*7–14*) documented the effectiveness of oral and topical psoralens for the photochemotherapy of vitiligo. Both 8-MOP and TMP have been effective orally with sunlight (*7–12*) or artificial UVA (*13, 14*) as the source of radiation.

Rarely has the issue of relative effectiveness of the various psoralens been addressed in a double-blind fashion. Seghal (*15*) reported a comparative study of 8-MOP, TMP, and psoralen in 89 patients with vitiligo. Among those

ABBREVIATIONS: 8-MOP = 8-methoxypsoralen; 5-MOP = 5-methoxypsoralen; TMP = 4,5′,8-trimethylpsoralen; UVA = UV radiation at 320–400 nm; PUVASOL = psoralen (orally administered) and solar UV radiation; J = joules.

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[4] We are particularly grateful to Drs. Rebecca Naylor, Russell Rowland, and John Wickman, and the Clinical Staff of the Bangalore Baptist Hospital, Bangalore, India, for their dedicated and enthusiastic support during this clinical trial; the Franco Indian Pharmaceutical Co., Bombay, India, for the psoralen; and Mac Laboratories, Bombay, for the 4,5′,8-trimethylpsoralen.

treated for 26 weeks or less, the skin of more patients was repigmented fully (13%) with 8-MOP, but the overall response was better with psoralen or TMP. These results are difficult for one to interpret in view of the fact that patients with vitiligo having vitiliginous macules involving 10% or more of the body area require 30 to 100 treatments extending over 6 to 9 months before 75 to 95% repigmentation can be achieved. In a 12- to 18-month study using artificial UVA, Parrish et al. (*14*) found 75% or greater repigmentation overall in 2 of 13 patients treated with 0.6 mg 8-MOP/kg compared with 4 or 13 treated with 0.6 mg TMP/kg. Three psoralens are presently used in many countries for the treatment of vitiligo: 8-MOP, TMP, and psoralen. Long-term comparative, double-blind studies with these compounds have neither been conducted nor reported. The question of which psoralen is most effective has never been addressed. Although 8-MOP and TMP were introduced between 1947 and 1960 for the treatment of vitiligo, they have never before been evaluated in a controlled, randomized, and double-blind manner under comparative conditions. To address this question of comparative effectiveness and toxicity, not only of several psoralens but also of several dosage schedules of each drug, we undertook a double-blind study in collaboration with Drs. Naylor, Rowland, Wickman, and the clinical staff at the Bangalore Baptist Hospital, Bangalore, India. For millions of patients with vitiligo throughout the world, and particularly in India, Pakistan, and other countries of the Far East, including the blacks of the United States, the search for an effective treatment or cure is intense, constant, endless, and even frantic. Vitiligo in India is a particularly disruptive problem for patients, and it spares no one regardless of age, sex, caste, or occupation. To persons with brown or black skin, vitiligo is a serious disfigurement problem that often leads to unhappiness, social segregation, lack of opportunities for advancement, deprivation of married life, and severe psychosocial problems. Although the disorder affects 1 to 2% of the population worldwide, with males and females probably equally affected, it appears to us that the incidence of vitiligo in India is high and approaching nearly 5% of the population according to outpatient attendance in clinics and the cosmetic concerns of females and males. With near missionary zeal, the clinical staff under the direction of Drs. Rowland, Wickman, Naylor have enthusiastically collaborated with us in addressing the effectiveness of PUVASOL in the treatment of this devastating, psychosocial problem. In addition, the melanogenic potential of various furocoumarins in normal human skin was also investigated.

MATERIALS AND METHODS

From the Outpatient Clinic of the Bangalore Baptist Hospital, 596 patients with vitiligo were enrolled in the study over a period of 6 months. Criteria for selection included over 1% vitiligo regardless of the site, age over 8 years, and absence of history of previous photosensitivity. Most patients had generalized vitiligo with 10 to 70% skin involvement with duration of 1 to 50 years. Males and females were equally represented. Pregnant and lactating women were excluded. The patients were randomly assigned to 1 of 8 treatment groups (*see* table 1). The dosage schedules included 0.3 and 0.6 mg 8-MOP/kg; 0.8, 1.8, and 3.6 mg TMP/kg; 0.3 mg 8-MOP plus 0.6 mg TMP/kg; 0.6 and 1.2 mg psoralen/kg; and placebo. Neither the investigators nor the patients were aware of the psoralen being ingested. The placebo group received lactose capsules for the first 9 to 12 months of the study. After a year of the study, patient pressure and desire for improvement, ethical considerations, and the social sensitivity and concern of the Bangalore Baptist Hospital staff led us to discontinue the placebo group, and the patients were given 3.6 mg TMP/kg. Hereafter, all patients of this group could be assured that they were on active drugs.

Patients were instructed to take the prescribed number of tablets (based on their respective weights) 2 hours before sun exposure between 10 a.m. and 2 p.m. at a location approximately 13° N latitude and at an elevation of 3,000 feet. They were to expose their skin for 30 to 45 minutes per side per treatment and to treat themselves thrice weekly on alternate days, *not* on 2 consecutive days. Initially and at 6-month intervals, each patient was photographed (with black and white film and with hospital unit number) in 8 positions (face and neck, anterior and

TABLE 1.—*Various treatment groups and psoralen dosage schedules used for patients with vitiligo*

Group	Drug dose, mg/kg	No. of patients at 2-yr follow-up
Combination low-dose 8-MOP plus medium-dose TMP	0.3 8-MOP plus 0.6 TMP	55
Low-dose 8-MOP	0.3	47
Medium-dose 8-MOP	0.6	49
Medium-dose TMP	0.8	39
High-dose TMP	1.8	61
Very high-dose TMP[a]	3.6	43
Medium-dose psoralen	0.6	35
High-dose psoralen	1.2	37
Placebo[b]	—	24

[a] Treatment was given for 1 yr only; this was initially the placebo group.
[b] After 1 yr of therapy, patients were given 3.6 mg TMP/kg.

posterior trunk and arms, anterior and posterior legs, and feet). The patients were seen in follow-up every 2 months at the outpatient department of the Bangalore Baptist Hospital and were evaluated extensively on a yearly basis. On each of our yearly visits, all patients were contacted to return to the hospital to be interviewed and examined by at least 2 investigators who examined the patients fully and compared their pigmentary status depicted in standardized photographs. The degree of repigmentation was rated visually against the photographs obtained at the first visit for enrollment in the study and at intervals of 6, 12, 18, and 26 months. The patients were interviewed in-depth for side effects, particularly nausea, headaches, dizziness or pruritus, and for evidence of acute and chronic phototoxic events. They were screened particularly for abnormal and unusual cutaneous lesions. The following laboratory tests were performed at the outset and then every 6 months: complete blood count, urinalysis, alkaline phosphatase, and serum glutamic-oxaloacetic transaminase.

The melanogenic potential of various furocoumarins in normal human skin was investigated in 12 fair-skinned Caucasian volunteers with skin types III and IV under laboratory conditions in the United States. Compounds listed in table 2 were applied topically to the skin of the backs of normal individuals in concentrations ranging from 5, 10, 25, 50, to 100 $\mu g/6\text{-cm}^2$ skin area for a test of their ability to cause erythema, edema, and vesiculation reactions. Each compound was tested on at least 3 volunteers. The nonphotosensitizing psoralens were applied at higher concentrations ranging from 50 to 500 $\mu g/6\text{-cm}^2$ skin area. Approximately 30 to 45 minutes after the psoralens were applied, the treated skin was exposed to UVA. To minimize severe toxicity, we kept the exposure dose of UVA for linear, photosensitizing furocoumarins at 2.5 to 4.0 J/cm^2 to provide a moderate degree of erythema (+ to ++). For nonlinear furocoumarins, the UVA exposure was increased to 12.0 J/cm^2. All subjects were treated and irradiated once, and the pigmentation response of the exposed sites was evaluated on days 7 and 10 after exposure. The minimal melanogenic dose in micrograms/square centimeter was estimated for each chemical.

RESULTS

The results of 2 years of therapy on 366 patients could be fully evaluated (tables 3, 4). The remainder of the original group of 596 was unavailable for annual or final evaluations, or both; over 70% of these 230 had simply been lost to follow-up. Some had moved to other cities, and others had job changes that made midday sun exposure impossible for them. Our best efforts to contact the "no shows" and the "dropouts" and obtain the reasons for their discontinuation were disappointingly hampered by poor, unreliable means of communication and primitive means of transportation.

Therapeutic Effectiveness of Psoralens

Among the 366 patients evaluated, the faces of 45% who were treated with either the combination dose of 8-MOP plus TMP or low-dose 8-MOP became fully repigmented, and nearly 60% achieved over 75 to 100% repigmentation of the head and neck (table 3). The chest, abdomen, and back repigmented nearly as well as and better than the arms and legs. Seventy-five percent or more repigmentation of all vitiliginous macules occurred in 38% of the patients who received the combination, 31% of those on low-dose 8-MOP, 29% of the admittedly small group on high-dose psoralen, but less than 17% for all TMP groups. Those on higher dosage schedules of psoralen and TMP generally did better than those on the lower dosage but still not as well as those who received 8-MOP or the combination of 8-MOP and TMP. The results of high-dose TMP (3.6 mg/kg) are available for only 1 year as this was the initial placebo group. The placebo group maintained for 1 year showed dismal repigmentation. After 2 years of therapy with 8-MOP, the patients receiving the lower dosage had done better than those on the higher dosage. The lips, hands, feet, palms, and soles were repigmented poorly in all treatment groups.

Initially, the patients receiving 0.6 mg 8-MOP/kg and the combination dose of 8-MOP and TMP showed a better response than those on the 0.3-mg 8-MOP/kg regimen, but after the second year, the rate of over 75% repigmentation response of 60–69% for the face, neck, and chest was much

TABLE 2.—*Percent response to PUVASOL in 366 patients treated for 2 yr*

Site	Drug and dosage, mg/kg															
	0.3 8-MOP and 0.6 TMP		0.8 TMP		1.8 TMP		3.6 TMP	0.3 8-MOP		0.6 8-MOP		0.6 Psoralen		1.2 Psoralen		Placebo
	yr		yr		yr		yr	yr		yr		yr		yr		yr
	1	2	1	2	1	2	1	1	2	1	2	1	2	1	2	1
Face	33	64	10	25	22	42	40	35	60	52	27	14	23	46	40	0
Neck	16	56	28	18	18	24	25	17	67	50	26	100	25	11	50	25
Chest	27	48	14	25	13	35	33	25	69	42	39	25	30	29	20	9
Arms	21	43	9	25	18	23	10	40	32	39	35	14	6	45	33	10
Legs	16	43	28	29	14	24	12	10	22	35	39	0	10	14	0	20
Hands	14	15	0	0	3	4	0	0	0	6	15	0	0	0	0	0
Total	24	38	19	17	11	14	9	19	31	23	25	7	3	9	29	0

[a] The placebo was only administered the first yr.

TABLE 3.—*Overall response of vitiligo patients treated for 2 yr or more*

Drug	Dosage/70-kg individual, mg	No. of patients	Overall repigmentation, %[a]
8-MOP and TMP	20 and 40	55	38
8-MOP	20	47	31
8-MOP	40	49	25
TMP	60	39	17
	125	61	14
	250	43[a]	19
Psoralen	40	35	7
	80	37	29
Placebo[b]		24	0

[a] Values indicate 75–95% repigmentation of the originally involved vitiliginous areas; this overall response is low because the nonresponding areas are also included in the calculations.

[b] Patients were treated for 9–12 mo only.

better than for the 0.6 mg 8-MOP/kg. Higher dosage TMP gave the best response of all for that drug. The combination of 8-MOP and TMP gave a better 2-year repigmentation response than did 0.8 or 1.8 mg TMP/kg alone. The 2-year response figures were not available for high dosages of TMP (3.6 mg/kg). However, low dosage 8-MOP gave comparable results except for the arms and legs. The results for high-dose psoralen exceeded those for the 0.6-mg/kg dose, but the results on the legs were disappointing. Only the combination and higher amounts of 8-MOP gave any degree (15%) of satisfactory response on the hands.

When all areas of involvement are considered together, the 2-year 75 to 95% repigmentation response appeared to be best with the combination group of 8-MOP plus TMP (38%), followed by 0.3 mg 8-MOP/kg (31%). These response figures include normally poorly responsive cutaneous areas of the hands, feet, palms, soles, lips, and nipples. The best criteria for success in general are the results with the face, neck, and trunk.

Side Effects of Various Psoralens

Subjective complaints registered most commonly included nausea, pruritus, and dizziness; headaches, eye discomfort, and vague gastrointestinal distress were noted by only a few patients. During the first year of the study, 51% of patients who had participated since the beginning reported no side effects. Pruritus was the most common (15%) and nausea and dizziness were next at 12%. However, 37% of the 30 patients in the placebo group had reported similar complaints of 10% nausea and 7% each pruritus and dizziness. Miscellaneous complaints were noted by a full 13% of the control group. In general, TMP was associated with the fewest complaints; 72% of patients on the lower dosage and 46% on the higher had no complaints. Dizziness was reported by 12%, pruritus by 9%, and nausea by 3% of 58 patients on TMP. Contrastingly, among the patients on 8-MOP, 20% reported nausea and pruritus with 8% noting dizziness and vague gastrointestinal discomfort. The combination regimen of low-dose 8-MOP and TMP appeared to enhance complaints with only 37% of 35 having none; a full 38% reported pruritus, 17% nausea, 6% dizziness, and 3% headaches. Of the patients given the psoralen, only the low-dose group was seen at this time; like those receiving the combination, a lower percentage (36%) had no complaints, with 14% reporting headaches and 9% each nausea, pruritus, and dizziness. Fourteen percent had miscellaneous complaints. With both 8-MOP and TMP, the incidence of side effects appeared dose related with nausea and pruritus occurring in 35% at the higher dose and 10% at the lower dosage schedule. A full 10% of the placebo group reported nausea and 7% itching.

Follow-up Studies

When all patients were examined after 1 year of PUVASOL therapy, none were found to have any abnormal cutaneous lesions on amelanotic skin or normally melanized skin. No keratoses or tumors were observed in any group. Many patients had evidence of chronic phototoxicity, i.e., erythema, desquamation, and thickening; these changes were exclusively localized to amelanotic skin. Uninvolved (normally pigmented skin) and remelanized skin were consistently normal in appearance. Of the group at large, over 45% had at least minimal phototoxic changes; similar changes were observed in about 50% of the combination and of the higher dose 8-MOP groups. Persistent erythema was present in 26% of the controls, 16% of the psoralen group, and 15% of the low-dose 8-MOP group. Increased thickening of the nonresponding amelanotic macules was also observed most frequently in patients receiving the combination 8-MOP and TMP and the medium dose 8-MOP. The figures were as follows: 42% as opposed to 15% placebo, 17 to 19% TMP, and 37% psoralen. None of the changes with either low-dose 8-MOP or TMP exceeded controls after a year of study. Only 22% of those treated with the combination had no phototoxic changes as opposed to about 60% among all other treatment groups and 68% who received placebos. Although a larger group of patients was interviewed and examined at the 26-month follow-up, only 366 patients who received PUVASOL therapy for 15 to 26 months could be carefully evaluated for repigmentation results and side effects. The placebo group had not been maintained for the last year of the study. The patients of the original placebo group had been scheduled to use 3.6 mg TMP/kg for nearly 14 months while following the same sun exposure protocol as the other groups. A significant percentage of patients continued to report side effects, but none were severe enough to necessitate limiting of their treatment with any of the psoralens.

Subjective complaints, registered by patients in all study groups, usually included nausea, gastrointestinal distress, pruritus, lightheadedness; headaches were the most common among those who received 0.6 mg 8-MOP/kg (particularly nausea and lightheadedness). For all groups, the prevalence of symptoms were dose related. Nausea was observed the least frequently with TMP even at the highest dosage schedules. Pruritus was also less common among those ingesting TMP than 8-MOP or psoralen. The occurrence of these complaints did not appear to limit the continuation of therapy.

Acute and chronic phototoxicity changes occurred with all dosage schedules for all psoralens but were most common with 8-MOP and least so with the lowest dosages of TMP and psoralen. Why these changes appeared less frequently to patients given the higher dosage of 8-MOP than to those receiving other amounts is unexplainable; it may be that severe phototoxicity in some of these patients may have influenced their continuation in the study; therefore, they were lost to follow-up. Although photosensitivity reactions were noted, the severe disabling type (e.g., erythema, edema, blistering, severe pruritus, etc.) were absent. No keratoses were noted at 26 months of treatment.

No consistent or persistent abnormalities of laboratory results were observed among the tests performed, i.e., complete blood count, urinalysis, alkaline phosphatase, and serum glutamic-oxaloacetic transaminase.

Melanogenic Potential of Various Furocoumarins in Normal Human Skin

The relative melanin-stimulating activity for various psoralens is shown in table 4. It is apparent that linear psoralens are more melanin-stimulating agents than are the nonlinear. Psoralens capable of evoking skin photosensitization responses appear to be more melanogenic than are nonphotosensitizing furocoumarins. When tested topically, the melanogenic effectiveness of TMP was the highest (TMP > psoralen > 8-MOP > 5-MOP). The nonphotosensitizing linear furocoumarins such as 3-carbethoxypsoralen, 8-hydroxypsoralen, 4′,5′-dihydro-8-methoxypsoralen, 5,8-dimethoxypsoralen, and the angular furocoumarins, such as angelicin (isopsoralen), 5-methylangelicin, and 4,5′-dimethylangelicin were either inactive or stimulated melanogenesis moderately. Those isopsoralens that showed enhanced photoconjugation with DNA (e.g., 5-methylangelicin and 4,5′-dimethylangelicin) stimulated more melanin pigmentation with minimal evidence of skin photosensitization than did those psoralens and isopsoralens that photoconjugated poorly with DNA and were nonphotosensitizing. Most of the monofunctional psoralens and isopsoralens appeared to be nonphotosensitizing or minimally photosensitizing. Because of these interesting findings, we had purposely selected TMP, 8-MOP, and psoralens for stimulating melanogenesis in patients with vitiligo.

DISCUSSION

Several useful conclusions could be drawn from the general clinical experience with PUVASOL in India. 1) Some areas of the skin responded better than others regardless of the type of psoralen used. The face, neck, trunk, upper arms, and legs have a favorable prognosis, whereas the hands, feet, palms, soles, lips, and nipples respond poorly. In our opinion, the face and neck tend to respond best; the chest, upper arms, lower arms, and legs in many cases respond as well. The back and abdomen are also comparable at a later stage. 2) A longer duration of therapy exceeding 9 to 12 months is correlated with an improved rate of repigmentation. The following are among many prognostic factors predictive of favorable response and are independent of the psoralen used: duration of therapy, regularity in the treatment, minimal evidence of phototoxicity, and the sites of vitiligo. The most limiting factors in therapy are the slow rate of repigmentation response and the normally difficult areas, including bony prominences. 3) The figures for total body repigmentation response, on the basis of calculations of the nonresponding areas, may be poor standards for comparison for they may potentially place undue weight on areas normally unresponsive to any psoralen photochemotherapy. The face, neck, trunk, upper arms, and legs are the best areas for one to judge comparative response among various psoralens. The dorsum of the hands, knuckles, lower legs (ankle and feet areas) and the areas of bony prominences were areas that responded poorly.

In general, the results with all 3 psoralen derivatives show that the face and neck are highly responsive areas, and the trunk and back are nearly as favorable as the face. However, the arms and legs seem a little less responsive but so are the palmar wrist, bony prominences of the elbow, and anterior lower leg; all of these areas repigment slowly

TABLE 4.—*Melanin-stimulating activity of certain linear and angular furocoumarins*

Compound tested	Concentration applied $\mu g/6\ cm^2$	UVA exposure dose, J/cm^2	Relative skin-photosensitizing activity[a]	Relative melanin-stimulating activity[b]	Type of psoralen
TMP	5–50	2–4	++++	++++	Linear
Psoralen	"	"	+++	++++	"
8-MOP	"	"	++	+++	"
5-MOP	5–100	"	++	+++	"
5,8-Dimethoxypsoralen	25–500	12.0	−	± to +	"
8-Isoamelenoxypsoralen	"	"	−	+	"
8-Hydroxypsoralen	"	"	−	±	"
4′,5′-Dihydro-8-methoxypsoralen	50–500	"	−	−	"
3-Carbethoxypsoralen	50–250	"	−	−	"
Isopsoralen (angelicin)	50–1,000	"	−	− to +	Angular
5-Methylangelicin	50–500	"	− to ±	+ to ++	"
4,5′-Dimethylangelicin	"	"	− to ±	+ to ++	"

[a] Amount of activity indicated is based on minimal phototoxic dose (micrograms and joules/square centimeter).
[b] Amount of activity indicated is based on minimal melanogenic dose (micrograms and joules/square centimeter).

and with difficulty in most patients. The anterior lower leg is capricious because in some affected individuals, this area may respond as well as any other isolated macules, whereas in others having an excellent response, the anterior lower leg is strikingly lagging in comparable responsiveness, particularly if marked leukotrichia is present. The hands, feet, palms, and soles (the 3 areas not shown in table 3, but included in the total figures) are unresponsive in all but exceptional instances. Interestingly, many patients who were responding well also showed increased pigmentation of the palms.

At the time this study began, we had included without bias patients with progressive vitiligo of the lips, digits of the hands and feet, and palmo-plantic areas. The vitiliginous macules in these areas are nonresponsive for all groups; an untoward number of such patients in any group could significantly contribute to a poor response being recorded for that group. In a randomized study such as this, inclusion of such patients should not present potential bias if the group were much larger. However, we believe that in this study, the results are best judged by examination of the repigmentation response of the nondistal and nonmucosal cutaneous areas.

If the poor responding areas are not taken into account in the assessment of the overall repigmentation response, then photochemotherapy with 8-MOP or TMP offers the best hope to patients in restoring the normal color of skin in such areas as the face, neck, chest, legs, thighs, arms, etc. We believe that the photochemotherapy of patients having generalized vitiligo with these 2 drugs can be an effective treatment given patient compliance and careful long-term follow-up. Depending on the sites of involvement, total or complete repigmentation in patients with 30% or more skin involvement is not impossible but often difficult; patients with 1 to 10% body involvement may show complete repigmentation in a reasonable amount of time provided that the areas involved do not include the tip of the fingers, knuckles, lips, ankles, and feet.

A clinician should always try to avoid moderate-to-severe phototoxicity. Persistent phototoxicity may cause the destruction of the surviving or proliferative melanocytes and contributes to an enhanced isomorphic (Koebner) phenomenon. Psoralens in general and TMP and 8-MOP in particular are potent skin photosensitizing agents that also produce reactive forms of oxygen (e.g., superoxide anions, singlet oxygen, and hydroxy radicals) and these reactive forms of oxygen are detrimental to cell membranes (Pathak MA: Unpublished observations).

Despite some chronic phototoxicity and occasional complaints of nausea and pruritus, the patients were generally pleased with their results. In the Bangalore study, 8-MOP was the most effective single drug, and the use of 0.6 mg 8-MOP/kg gave the best results after 1 year but fell behind the group receiving 0.3 mg 8-MOP/kg in the second year; our inability to communicate with the dropouts made it impossible to explain these observations. Because the higher dosage group (0.6 mg/kg) included a number of patients who had done better in the first year of therapy than in the second, particularly in uncovered areas of the face, chronic isomorphic responses resulting from damage to cell membranes cannot be excluded in an explanation of the decline. The addition of TMP to the low-dosage 8-MOP regimen (combination therapy with 8-MOP plus TMP) seemed to improve the response in the first 9 months of therapy, particularly on the arms and legs. It is certainly possible that long-term usage of high-dosage TMP would give better results than would the 0.8 and 1.8 mg/kg schedules. Additional study seems warranted. The parent furocoumarin, psoralen, at 0.6 mg/kg gave poor results and was not an effective therapeutic agent; doubling the dosage did not appear to have a significant effect on repigmentation in a limited number of patients. This psoralen appears to be least effective in stimulating the repigmentation of vitiliginous skin.

Although the changes of chronic phototoxicity were observed, strikingly absent at all observations was evidence of severely disabling photosensitivity, i.e., marked erythema, edema, blistering, etc. Two patients did report blistering, but this is a small percentage considering the number of patients, the number of treatments, and the latitude. Furthermore, no actinic keratoses or skin tumors were apparent at 26 months of treatment. However, by the fourth year of follow-up when 220 patients, most of whom have been treated for over 48 months, were recalled, 13% (29 of 200 actually examined) developed at least 1 keratotic lesion in vitiliginous macules; most of these keratoses were located on the lower legs (*16*). Fifteen of the 29 patients had more than 1 lesion and 5 had multiple lesions of several different morphologic types. These patients had continued to expose their vitiliginous macules to solar radiation with the hope that these areas would gradually repigment in the same manner as other areas which had responded favorably. Clinically, these lesions were keratotic papules, actinic keratosis-like macules, smooth dome-shaped papules, and lichenoid porokeratotic-like papules. Lesions were found in patients treated with each of the psoralens used in the study. However, those patients given 3.6 mg TMP/kg had the highest incidence (32%) of lesions. No 1 morphologic type could be associated with any 1 psoralen or treatment regimen except that the dome-shaped papules were found only in the 3.6-mg TMP/kg group. Histologically, the lesions showed benign papillary epidermal hyperplasia with hyperkeratosis, papillary actinic keratosis, and atrophic actinic keratosis..Only 7 of the lesions were consistent with actinic keratosis, and the degree of atypia in all lesions was mild; none were present after 1 to 2 years of therapy. Unfortunately, no age-matched control population received similar solar irradiation without prior psoralen ingestion. Many patients had also used prior remedies we found difficult to identify, but some admitted to have used topical 1% psoralen ointment. No lesions were observed on normally melanized skin or on remelanized skin of vitiliginous macules. No squamous cell carcinomas, basal cell carcinomas, or melanomas were observed.

Of the 220 patients who developed these lesions after 4 years of therapy, 29 were older, had their vitiligo longer, received the psoralen treatment longer, and generally responded better to treatment than the group at large. Many had achieved over 75% repigmentation everywhere but the hands and the lower legs (sites of the keratoses).

The mechanism of repigmentation by psoralens in vitiligo is unclear. They have been shown to stimulate

melanogenesis in normal skin (*17–21*); this results from a twofold to threefold increase in the number of melanocytes which become hypertrophic and increasingly arborized (*22, 23*). In addition, tyrosinase activity, synthesis of melanosomes in melanocytes, and transfer of these granules to keratinocytes are increased (*23, 24*). Repigmentation, which appears to result from the migration of melanocytes from UVA-activated follicular reservoirs of hair bulb melanocytes to the dermo–epidermal junction, has been demonstrated with scanning electron microscopy (*25*); melanocytes appear to be ascending the follicular apparatus to repopulate the dermo–epidermal junction.

Increased melanin pigmentation must be related to photoconjugation with pyrimidine bases in DNA to form monofunctional and bifunctional photoadducts between DNA base pairs (*22*). This may retard melanocyte cellular division in the hair bulbs and prolong the exposure of the melanocyte cell surface to the action of melanocyte-stimulating hormone and adenylate cyclase in the G_2 phase of the cell cycle (*26*). The melanocyte-stimulating hormone is then more likely to stimulate the tyrosinase activity. The increased activation of tyrosinase may also follow decreased inhibition of tyrosinase activity by sulfhydryls (e.g., glutathione, cysteine) because of the oxidation of these sulfhydryl groups by a photosensitization mechanism. Within 48 to 72 hours, the melanocytes appear to undergo repair of the damaged DNA by excision repair of the pyrimidine–psoralen photoadducts. Melanocytes thereby replicate their DNA, and mitotic cellular replication begins. This results in an increased number of functioning melanocytes with enhanced tyrosinase activity stemming from the increased synthesis of protein in the proliferating melanocytes. The enhanced number of melanocytes leads to increases in the synthesis of melanosomes and in the transfer of melanosomes from melanocytes to keratinocytes. The increased arborization of dendrites contributes to greater transfer of melanosomes. Thus psoralens and UVA appear to enhance repigmentation of amelanotic skin by the 1) stimulation of melanocyte mitosis, 2) proliferation of melanocytes, 3) enhancement of tyrosinase activity, 4) increased melanosomal formation and transfer, and 5) induction of melanocyte migration.

The presence of a lymphocytic infiltrate at the margins of active vitiligo lesions suggests another role for psoralens. When given with UVA, they may also exert lympholytic effects on the host to permit a favorable cutaneous environment for melanocyte migration and viability.

Successful repigmentation of vitiliginous skin in motivated patients is a realistic objective, but long-term therapy is required. Between 15 and 25 treatments are generally necessary before perifollicular repigmentation is initially apparent. Between 100 and 300 treatments are usually required for complete repigmentation of the face, neck, trunk, and upper arms and upper legs, though often the face seems to repigment faster. With about 85–90% surety, macules which are completely repigmented should stay so even if therapy is discontinued (*27*).

In work still in progress in our laboratory, suberythemogenic doses of UVA appear to be adequate to induce repigmentation, and no accelerated response is found in those patients whose therapeutic doses are maintained at minimal or 1+ erythema levels. There may be a threshold above which chronic phototoxicity perpetuates vitiligo by the Koebner phenomenon. This is one possible explanation for the poorer results obtained in the second year with 0.6 mg 8-MOP/kg than with 0.3 mg 8-MOP/kg in the Bangalore study.

We concluded that 8-MOP appears to be the most effective psoralen currently available for repigmentation. However, it is usually much more phototoxic than is orally administered TMP. For this reason, TMP is often recommended for fair-skinned individuals in an unsupervised environment. The less phototoxic compound is often selected in the interest of avoiding potentially severe phototoxic events and associated complications resulting from the Koebner phenomenon.

Parenthetically, TMP is also extremely phototoxic for some individuals; therefore, individualization of therapy is necessary. None of the 3 psoralens can overcome the limitation of poor response of the fingers, toes, palms, soles, nipples, ankles, and lips. Except for individuals of skin types V and VI, the digits, palms, and soles of persons with skin types I through IV probably should be prophylactically shielded from UVA exposure to minimize the potentially harmful effects of UV exposure.

Despite the substantial number of regular treatments with psoralen and sunlight, even at 4 years of follow-up, patients neither developed cutaneous cancers nor developed any abnormal liver function values. Antinuclear antibody determinations were not available at the Bangalore Baptist Hospital at the time of the study.

Our recommendations for the photochemotherapy of vitiligo are:

For fair-skinned individuals of skin types I, II, and III (who burn easily with sunlight exposure), 0.6 mg TMP/kg is recommended to avoid potential, severe phototoxicity associated with oral 8-MOP therapy.

For pigmented individuals of skin types IV, V, and VI (brown- or black-skinned patients who do not sunburn easily), 0.3 to 0.6 mg 8-MOP/kg is recommended to stimulate increased pigmentation. If the repigmentation response is not visible after 25 to 30 treatment sessions and before a given psoralen is considered a failure, the patient should be switched to either 8-MOP or TMP or a combination of the 2 drugs (0.3 mg 8-MOP and 0.6 mg TMP/kg).

Individuals living in sunny locations with high-intensity UV radiation (0–30° North and South latitude) should receive gradual increments of exposure. The initial 10 or 15 minutes total exposure is gradually increased to a maximum of 1 hour (both sides) making sure not to burn the skin on any treatment day. Usually a maximum of 30 minutes of exposure per side per treatment is adequate for long-term therapy. The minimum frequency of treatment should be at least twice per week or every third day; maximum treatments are thrice per week on alternate days. Persistent erythema of vitiligo patches should be avoided. Some patients, while showing repigmentation of vitiliginous macules, may also continue to develop new lesions in the course of treatment. This is not necessarily a harbinger of poor response or failure but should alert the physician to the problems with light exposure or dosage schedule. The

treatment with oral psoralen and sunlight should be accompanied by the proper shielding of the eyes with UV-filtering glasses for 24 hours after drug ingestion if the patient remains outdoors or near a bright, sunlit window indoors.

REFERENCES

(*1*) FITZPATRICK TB, PATHAK MA: Historical aspects of methoxsalen and other furocoumarins. J Invest Dermatol 32:229–231, 1959

(*2*) PATHAK MA, DANIELS F, FITZPATRICK TB: The presently known distribution of furocoumarins (psoralens) in plants. J Invest Dermatol 39:225–239, 1962

(*3*) FAHMY IR, ABU-SHADY H: Pharmacognostical study and isolation of crystalline constituents, ammoidin. Q J Pharm Pharmacol 20:281–290, 1947

(*4*) EL MOFTY AM (ed): Vitiligo and Psoralens. New York, Oxford: Pergamon Press, 1968

(*5*) KUSKE H: Experimentelle Untersuchungen zur Photosensibilisierung der haut durch Pflanzlich Wirkstoffe. Arch Dermatol Syph (Berlin) 178:112–123, 1939

(*6*) UHLMANN E: Die Behandlung der Vitiligo und die Voraussetzungen fuer die kunstliche Pigmentierung der Haut. Med Welt 8:226–228, 1934

(*7*) EL MOFTY AM: A preliminary clinical report on the treatment of leukoderma with *Ammi majus linn.* J R Egypt Med Assoc 31:651–665, 1948

(*8*) SIDI E: Dyschromies et Vitiligo. Expansion Scientifique Francaise (Pequignot H, ed). Paris: Expansion, 1957

(*9*) ELLIOT JA JR: Clinical experiences with methoxsalen in the treatment of vitiligo. J Invest Dermatol 32:311–313, 1959

(*10*) AFRIDI J, FUCRON J: Treatment of vitiligo with topical trimethylpsoralen and sunlight. Br J Dermatol 84:151–156, 1971

(*11*) SEGHAL VN, REGE VL, VADIRAJ SN: Topical neosoralen (TMP) in vitiligo. Australas J Dermatol 12:101–107, 1971

(*12*) ———: Effectiveness of trioxsalen therapy for vitiligo. Arch Dermatol 110:957–958, 1974

(*13*) BLEEHAN SS: Vitiligo treatment with trimethylpsoralen. Br J Dermatol 84:186–188, 1971

(*14*) PARRISH JA, FITZPATRICK TB, SHEA C, et al: Photochemotherapy of vitiligo. Use of orally administered psoralens with a high intensity longwave ultraviolet light (UVA) system. Arch Dermatol 112:1531–1534, 1976

(*15*) SEGHAI VN: A comparative clinical evaluation of TMP, psoralen, and 8-MOP in treating vitiligo. Int J Dermatol 14:205–208, 1975

(*16*) MOSHER DB, PATHAK MA, HARRIS TJ, et al: Development of cutaneous lesions in vitiligo during long-term PUVA therapy. J Invest Dermatol 74:259, 1980

(*17*) ARNOLD HL JR: Effect of methoxsalen on inability to tan. J Invest Dermatol 32:341–342, 1959

(*18*) FITZPATRICK TB, HOPKINS CE, BLICKENSTAEE DD, et al: Augmented pigmentation and other responses of normal skin to solar radiation following oral administration of 8-methoxypsoralen. J Invest Dermatol 25:187–190, 1955

(*19*) KANOF ND: Clinical experience with effect of oral 8-methoxypsoralen in the pigmentary responses of skin. J Invest Dermatol 32:343–344, 1959

(*20*) IMBRIE D, DANIELS F JR, BERGERON L, et al: Increased erythema threshold six weeks after a single exposure to sunlight plus oral methoxypsoralen. J Invest Dermatol 32:331–340, 1959

(*21*) STEGMAIER OC: The use of methoxsalen in suntanning. J Invest Dermatol 32:345–349, 1959

(*22*) PATHAK MA, KRÄMER DM, FITZPATRICK TB: Photobiology and photochemistry of furocoumarins (psoralens). *In* Sunight and Man: Normal and Abnormal Photobiologic Responses (Fitzpatrick TB, Pathak MA, Harber LC, et al, eds). Tokyo: Univ Tokyo Press, 1974, pp 335–368

(*23*) TODA K, NAKAYAMA Y, MORIKAWA F: Electron microscopic observations in human skin after psoralen photosensitization. *In* Sunlight and Man: Normal and Abnormal Photobiologic Responses (Fitzpatrick TB, Pathak MA, Harber LC, et al, eds). Tokyo: Univ Tokyo Press, 1974, pp 419–430

(*24*) JIMBOW K, PATHAK MA, SZABO G, et al: Ultrastructural changes in human melanocytes after ultraviolet radiation. *In* Sunlight and Man: Normal and Abnormal Photobiologic Responses (Fitzpatrick TB, Pathak MA, Harber LC, et al, eds). Tokyo: Univ Tokyo Press, 1974, pp 195–216

(*25*) ORTONNE JP, SCHMITT D, THIVOLET J: PUVA induced repigmentation of vitiligo: Scanning electron microscopy of hair follicles. J Invest Dermatol 74:40–42, 1980

(*26*) COHEN SR, BURKHOLDER DE, VARGA JM, et al: Cell cycle analysis of cultured mammalian cells after exposure to 4,5′,8-trimethylpsoralen and long-wave ultraviolet light. J Invest Dermatol 76:409–413, 1981

(*27*) KENNEY JA JR: Vitiligo treated by psoralens: A long-term study of the permanency of repigmentation. Arch Dermatol 103:475–480, 1971

DISCUSSION

T. B. Fitzpatrick: Dr. David Mosher was a collaborator in the study that was done in India on the use of psoralens in treatment of patients with vitiligo.

D. M. Carter: I would like to be the first to congratulate you, Dr. Mosher, but I also want to ask you a question. Can you transfer this kind of experience to other parts of the world? The problem with doing so relates to altitude, latitude, and other geographic factors that affect sunlight. Could you comment from your experience on the use of these agents in temperate zones, i.e., with sunlight and with artificial sources?

D. B. Mosher: I would simply say that we now use tripsoralen for individuals with skin types II, III, and IV in the northern latitudes. We are comfortable with it and use sunlight as a source to repigment. By and large, results are the same. With indoor PUVA and using 8-MOP or TMP, we found treatment is more controlled and can be given all year. Orally administered TMP is not as phototoxic as is 8-MOP.

K. Smith: The story we had heard was that vitiligo has been treated topically with psoralens for centuries with absolutely no evidence of any bad effects. Now we are seeing bad effects when oral treatment is given. Has any decision been made regarding return to topical treatment of vitiligo? What is the status of this now?

Fitzpatrick: Topical treatment in mice (8-MOP and UVA) is carcinogenic; orally administered 8-MOP is not as carcinogenic. I think that vitiligo is a simple toxicity problem because the number of treatments is limited. If patients do not respond, we stop treatment. It is quite different from treatment for psoriasis when PUVA can be administered for 5 years. These people with vitiligo never wear sunglasses and they are out all day long. I think topical

psoralen is going to be much more photocarcinogenic in animals than the oral because it is much more difficult to control; as you heard, the phototoxicity remains for a long time.

J. H. Epstein: What about recurrence?

Fitzpatrick: We have no data about recurrence, but those patients who get 100% repigmentation remain repigmented for several years.

Mosher: Dr. Kenney studied that retrospectively some years ago and reported that 90% of the individuals who had completely remelanized a macule would retain it for up to 14 years. I do not have that kind of quantitative experience, but my impression is that the retention is in the vicinity of 80–85%. We consider PUVA contraindicated in children, unless the psychosocial reason to treat them is compelling. It is never the treatment of choice for children under 12 years of age.

Improvement of the Efficacy and Safety of Oral Methoxsalen Photochemotherapy[1]

Khosrow Momtaz-T *and* John A. Parrish[2]

ABSTRACT—To improve efficacy and safety of psoralen plus UV radiation at 320–400 nm (PUVA), investigators designed different therapeutic approaches. Our goals are to minimize the acute risks and lower the long-term hazards. Some of these therapeutic approaches focus on dosimetry and intensity of PUVA and some on combining PUVA with other therapeutic modalities. Both approaches try to minimize the total number of treatments and to decrease the amount of UVA radiation delivered to the patient and, as a result, decrease the total skin insult. — Natl Cancer Inst Monogr 66: 175–178, 1984.

To improve PUVA, one must constantly gather more information and be both creative and critical when evaluating clinical studies. The clinician must learn from the basic scientist, and the basic investigator should understand the goals, assumptions, and approach of the clinician. Communication about therapeutic applications of psoralens is seriously hampered by the fact that we do not actually know the exact molecular and cellular mechanisms for any of the therapeutic effects. The mechanisms we do propose are vague, descriptive, and cyclic restatements of the obvious. For one to say that the mechanism of action in psoriasis is inhibition of cell proliferation is simply to restate that replication kinetics are normalized by treatment. A claim that the mechanism of PUVA therapy of mycosis fungoides is selective photocytotoxicity of abnormal cells is only a reminder that such cells are present before treatment and absent afterward. A statement that PUVA stimulates migration and mitosis of melanocytes in vitiligo only defines the histologic findings before and after treatment. It is also not unusually helpful for a clinician to say that the therapeutic effect of PUVA in polymorphous light eruption or lichen planus is by lymphocytotoxic or photoimmunologic mechanisms.

We simply cannot give the best guidance to basic research because we do not know how the treatment works. This problem is confounded by unclear ideas of molecular mechanisms of side effects. However, we can attempt to state clearly our working hypotheses, assumptions, and our goals in therapy.

We assume that, for PUVA to be effective, phototoxic doses must be achieved. Photon-induced cell injury must occur. Usually, but not always, enough cell injury occurs to induce inflammation in normal skin as manifested by delayed erythema. The absence of photon-induced erythema does not necessarily mean the absence of cell injuries. For example, suberythemogenic doses of UVB have been shown to cause DNA damage (*1*), dyskeratotic cells (*2*), melanogenic repair response (*3*), and a decrease in erythema threshold (*4*).

We also assume that the acute risks (primarily the signs and symptoms of inflammation) are dose related and that careful dosimetry allows us to achieve phototoxic therapeutic doses with tolerable, acceptable, controllable side effects. Largely through empirical trials, protocols have been designed to minimize acute effects. Variables to be adjusted include the frequency of treatment, drug dose, preirradiation period (time between drug ingestion and UVA exposure), UVA dose, and the schedule of increases in dose of radiation as melanization and skin tolerance increase.

Finally, we assume that the long-term hazards (skin cancer, actinic degeneration, immunologic and ophthalmologic effects) are also dose related and are of acceptable magnitude for many patients with severe psoriasis. Long-term side effects are thought to be related to the number of treatments, the degree of phototoxicity at each treatment, and to a cumulative effect from repeated phototoxic insult roughly quantified by the total cumulative UVA dose. Therefore, it is obvious that the clinician has a strong obligation to minimize the total amount of treatment. Psoriasis is a life-long disease and the therapist must look beyond the most certain clearing schedule to consider the total skin insult over the long view.

APPROACHES TO IMPROVED CLEARING SCHEDULES

A few examples of different approaches to improved clearing schedules will be listed. Others exist and we hope new ones will be developed.

"More is Less"

This technique, widely used in Europe (*5*), is designed to administer maximum doses and frequency of treatment early in the course of therapy before PUVA-induced melanization, thickened stratum corneum, and other un-

ABBREVIATIONS: PUVA = psoralen plus UV radiation at 320–400 nm; UVB = UV radiation at 290–320 nm; J = joules.

[1] Presented at the Conference on Photobiologic, Toxicologic, and Pharmacologic Aspects of Psoralens held in Research Triangle Park, North Carolina, March 1–3, 1982.

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known factors reduce the effect of treatment. The advantage appears to be clearing in fewer treatments and therefore at lower total cumulative doses. Because it is usually necessary that the UVA dose be increased constantly throughout the treatment program, the omission of the late treatments has significant effect on reduction of cumulative UVA dose. The disadvantages of this protocol include inconvenience of more frequent treatments and the necessity for close observation for acute side effects.

Plateau

After patients begin to improve significantly, the UVA dose is no longer increased but is held constant throughout the clearing phase. Although this may make a greater number of treatments necessary, the UVA dose per treatment is decreased.

Insurance Hits

After clearing has been achieved, the patient is continued on the clearing schedule for several days or weeks before treatment is discontinued or maintenance therapy is initiated. This approach is believed to increase remission time or to decrease treatment requirements during maintenance.

COMBINATION THERAPIES

The proponents of combination therapies make certain assumptions: Two therapies most likely have different therapeutic mechanisms or at least have different long-term toxicity. They hope that the therapeutic effects will be additive or synergistic while the toxicities are neither. Combined therapy seeks to use less of either component of therapy and thereby minimize toxicity from either agent and reduce total toxicity.

Combination of Psoralen Photochemotherapy and Topical Agents

Anthralin

Anthralin (1,8-dihydroxyanthranol) is a synthetic aromatic hydrocarbon which may exert its effect by interacting with nucleic acids (mitochondrial DNA in particular), blocking protein synthesis, lowering the mitotic rate (*6*), decreasing the enzymes involved in carbohydrate metabolism, and inhibiting skin respiration in vitro, possibly by blocking oxygen uptake (*7*). Anthralin has been used alone or in combination with other antipsoriatic therapies for many years to control and treat psoriasis.

Willis and Harris (*8*) combined topically applied methoxsalen solution photoactivated by UVA radiation with a low-strength anthralin paste (0.4%) for the treatment of resistant psoriasis. Initial trials resulted in complete clearing at test sites within 1 to 3 weeks, whereas recalcitrant lesions at control sites receiving anthralin alone, anthralin plus methoxsalen, anthralin plus UVA, anthralin plus sun lamp, anthralin plus UVA and sun lamp, methoxsalen alone, methoxsalen plus UVA, methoxsalen plus sun lamp, methoxsalen plus UVA and sun lamp, UVA alone, sun lamp alone, and UVA plus sun lamp remained unaffected.

Morison et al. (*9*) studied the combination of oral psoralen photochemotherapy and topical application of anthralin. Different strengths of anthralin (0.25, 0.5, and 1% anthralin ointment and 0.4% anthralin in Lassar's paste) were used. In 1 group of patients, anthralin application began 6 weeks before exposure to PUVA, and in another, both treatments commenced at the same time. In both groups, the combination therapy required fewer PUVA exposures and less UVA dose (14 treatments, 12 J/cm^2 UVA dose at clearing for the first group, and 15 treatments, 12 J/cm^2 UVA dose at clearing for the second group) compared with control groups who received PUVA alone (23 treatments, 17 J/cm^2 UVA dose at clearing). The disadvantages of anthralin use on outpatients were mainly poor patient compliance resulting from messiness of anthralin, irritation, and a Koebner reaction secondary to its use.

Corticosteroids

Topical corticosteroids are used widely in the treatment of psoriasis. Their cost, production of both local and systemic side effects, and gradual loss of effectiveness limits their prolonged use in generalized severe psoriasis.

A combination of PUVA and topically applied corticosteroids has been shown to be superior to either treatment alone (*9–11*). In a controlled study, Morison et al. (*9*) used fluocinolone acetonide under plastic occlusion in combination with PUVA. This regimen resulted in a rapid clearance phase of 14 treatments compared with 23 treatments required by a control group who received only PUVA. The UVA dose at clearing was 11 J/cm^2 for combination therapy compared with 17 J/cm^2 for controls. Hanke and co-workers (*10*) compared the combination of betamethasone valerate plus PUVA to PUVA alone. Ten of 12 patients who participated in this bilateral comparison study cleared with combination therapy faster than with PUVA alone. The other 2 patients had equal clearing on both sides. Compared with patients treated with PUVA alone, Morison and associates (*9*) noticed higher relapses in the maintenance phase. Hanke et al. (*10*), who used betamethasone valerate plus PUVA, and Gould and Wilson (*11*), who administered clobetasol propionate plus PUVA in a bilateral comparison study and demonstrated more rapid clearing of psoriasis with topically applied corticosteroids in conjunction with PUVA, found no tendency to rebound in the maintenance phase. The plastic occlusion technique used by Morison et al. might explain the tendency to rebound.

Tar

Coal tar has been used alone or in combination with other therapeutic modalities in controlling psoriasis. Morison et al. (*9*) used the combination of refined tar gel (Estar) and PUVA. Nineteen subjects who participated in this controlled study were compared with 18 who received PUVA alone. The authors concluded that combination PUVA and tar did not produce any significant benefit over PUVA alone.

Psoralen Photochemotherapy and Systemic Agents

Retinoid

Topical and systemic retinoids [Ro10-9359 (Tigason or etretinate)] and vitamin A acid have been prescribed for

many years in the treatment of numerous different dermatoses. They affect the differentiation of epithelial cells. Some of these compounds carry the risk of side effects, including dry mucous membrane, hair loss, and liver damage. The analogs of vitamin A acid or retinoic acid have been effective in the treatment of psoriasis with increased effectiveness and safety, but side effects continue to be bothersome clinical problems (*12, 13*).

To reduce total dose and diminish side effects of PUVA and aromatic retinoid, some have given combined treatments (*14–17*). Fritsch et al. (*14*) treated 36 psoriatic patients and 12 with resistant palmar-plantar psoriasis with a combination of aromatic retinoid and PUVA. The results showed that combination therapy is superior to either therapy alone. Prior administration of retinoids for 7 to 10 days followed by addition of PUVA was most successful in their reducing the dosage of both therapeutic modalities and the number of PUVA exposures to achieve clearance. This combination reduced the number of PUVA irradiations by more than 50% and the total applied cumulative UVA energy by 75%, compared with the standard PUVA regimen. Patients with the palmar-plantar psoriasis or previous PUVA failures also responded to combination therapy.

Methotrexate

Methotrexate, a folic acid antagonist, has been an extremely effective and useful drug in the management of severe, recalcitrant psoriasis. Due to the potential side effects, it should be administered to selected psoriatic patients. The major toxicity from methotrexate is its effect on the liver, possibly leading to cirrhosis. The other adverse effects are rare and include pneumonitis, pulmonary fibrosis, kidney alterations, and idiosyncratic hematologic reactions.

Morison and associates (*18*) used the combination of methotrexate and PUVA for the treatment of 30 psoriatic patients. The patients first received 3 weekly courses of methotrexate (5 mg orally every 12 hr × 3 doses). After completion of the third course, PUVA therapy was begun and methotrexate was continued. When clearance was achieved, methotrexate was stopped, but they continued PUVA therapy to achieve a satisfactory maintenance regimen. This combination significantly reduced the total PUVA dose. Twenty-eight of 30 patients achieved clearance in an average of 9.3 PUVA exposures. The average UVA dose at clearing was about 6.2 J/cm^2. None of the treated patients showed any side effects thought to be related to methotrexate. The mean total methotrexate dose was only 93 mg. The main problem of this combination treatment was a tolerable but symptomatic subacute phototoxicity, which was observed in 8 of the 30 patients.

Combination of Psoralen and Ultraviolet Radiation at 290–320 and 320–400 nm

Both UVB and PUVA are effective treatments for psoriasis. When we (*19*) combined these 2 modalities in a protocol utilizing full doses of each, psoriatic lesions were cleared in one-half the number of treatments usually required for each treatment alone.

Thirty-eight of 40 patients with chronic psoriasis achieved clearance with PUVA and UVB in an average of 11 treatments in a time scale of 3.8 weeks. The patients were exposed to both forms of treatment on the same visit three times a week. Both UVB and UVA exposures took place 2 hours after ingestion of 0.6 mg methoxsalen/kg. Bilateral comparison studies and comparison of combination therapies with previously reported data in PUVA alone (*20*) and UVB alone (*21*) showed that a combination was superior to each treatment alone. When UVB in combination therapy was compared with previously published results on UVB with the same protocol, it was noted that the combination required one-half the number of treatments, one-third of the UVB dose at clearing, and one-fifth the cumulative UVB dose. When PUVA in combination therapy was compared with that given in a multicenter PUVA study (*20*), we found the number of treatments and cumulative PUVA dose needed to achieve clearance were almost one-half the same required by PUVA alone. Also noted was that if the combination is compared with maximally aggressive regimens of UVB or PUVA used, the advantages of combination were less impressive.

This combination has the advantage of 1) the cumulative dose for PUVA and UVB is decreased; 2) the patients can enter on a maintenance regimen of UVB or PUVA at lower doses because their dose at clearance is markedly reduced; and 3) problem patients who have not responded to PUVA alone or UVB alone often benefit from this combination therapy. Sparing effects of PUVA or UVB may or may not be a real long-term advantage depending on whether the long-term dose effects of the 2 treatments are additive or not or synergistic.

Cyclic Treatments

Phototherapy, oral psoralen photochemotherapy, and methotrexate are effective treatments for psoriasis. All these treatment modalities carry the risk of long-term toxicity. Long-term risks of UVB and PUVA include actinic degeneration, premalignant mutations in epidermis, and skin cancer in certain susceptible individuals. Prolonged use of methotrexate may lead to liver damage. The long-term risks are more likely related to cumulative dose and are probably at least partially reversible over time. Physicians presently change the treatments when patients begin to respond poorly or when long-term toxicity begins to be evident. It may be wise for a clinician to plan cycling of therapies from the beginning and cycle the 3 treatment modalities before obvious difficulties arise.

REFERENCES

(*1*) Gschnait F, Brenner W, Wolff K: Photoprotective effect of a psoralen-UVA-induced tan. Arch Dermatol 268:181–188, 1978

(*2*) Kaidbey KH, Grove GL, Kligman AM: The influence of longwave ultraviolet radiation on sunburn cell production by UVB. J Invest Dermatol 73:243–245, 1979

(*3*) Kaidbey KH, Kligman AM: The acute effects of longwave ultraviolet radiation on human skin. J Invest Dermatol 72:253–256, 1978

(*4*) Parrish JA, Zynoun S, Anderson RR: Cumulative effects of repeated subthreshold doses of ultraviolet radiation. J Invest Dermatol 76:356–358, 1981

(5) Wolff K, Fitzpatrick TB, Parrish JA, et al: Photochemotherapy for psoriasis with orally administered methoxsalen. Arch Dermatol 112:943–950, 1976

(6) Zetterberg C, Swanbeck C: Studies on dithranol and dithranol-like compound mutagenicity. Acta Derm Venereol (Stockh) 51:41–44, 1971

(7) Hodge L, Comaish JS: Psoriasis: Current concepts in management. Drugs 13:288–296, 1977

(8) Willis I, Harris RD: Resistant psoriasis: Combined methoxsalen-anthralin therapy. Arch Dermatol 107:358–362, 1973

(9) Morison WL, Parrish JA, Fitzpatrick TB: Controlled study of PUVA and adjunctive topical therapy in the management of psoriasis. Br J Dermatol 98:125–132, 1978

(10) Hanke CW, Steck WD, Roenigk HH Jr: Combination therapy for psoriasis: Psoralens plus long-wave ultraviolet radiation with betamethasone valerate. Arch Dermatol 115:1074, 1979

(11) Gould PW, Wilson L: Psoriasis treated with clobetasol propionate and photochemotherapy. Br J Dermatol 98: 133–136, 1978

(12) Van der Rhee HJ, Tijssen JG, Herrmann WA, et al: Combined treatment of psoriasis with a new aromatic retinoid (Tigason) in low dosage orally and triamcinolone acetonide cream topically: A double-blind trial. Br J Dermatol 102:203–212, 1980

(13) Lassus A: Systemic treatment of psoriasis with an oral retinoic acid derivative (Ro10-9359). Br J Dermatol 102:195, 1980

(14) Fritsch PO, Hönigsmann H, Jaschke E, et al: Augmentation of oral methoxsalen photochemotherapy with an oral retinoic acid derivative. J Invest Dermatol 70:178–182, 1978

(15) Orfanos CE, Pullman H, Sterry W, et al: Retinoid-PUVA (RePUVA): Systemische Kombinations behandling bei Psoriasis. Z Haukr 53:494, 1978

(16) Heidbreder G, Christophers E: Therapy of psoriasis with retinoid plus PUVA. Clinical and histological data. Arch Dermatol Res 264:331, 1979

(17) Lauharanta J, Juvakoski T, Lassus A: A clinical evaluation of the effects of an aromatic retinoid (Tigason), combination of retinoid and PUVA, and PUVA alone in severe psoriasis. Br J Dermatol 104:325–332, 1981

(18) Morison WL, Momtaz-T K, Parrish JA, et al: Combined methotrexate–PUVA therapy in the treatment of psoriasis. J Am Acad Dermatol 6:46–51, 1981

(19) Momtaz-T K, Parrish JA: Combination of PUVA and UVB (PUVA/B) in the treatment of psoriasis vulgaris: A bilateral comparison study. J Am Acad Dermatol 10: 481–485, 1984

(20) Melski JW, Tanenbaum L, Parrish JA, et al: Oral methoxsalen photochemotherapy for the treatment of psoriasis: A cooperative clinical trial. J Invest Dermatol 68: 328–335, 1977

(21) Adrian RM, Parrish JA, Momtaz-T K, et al: Outpatient phototherapy of psoriasis. A new protocol for thrice weekly treatment. Arch Dermatol 117:623–626, 1981

Effectiveness of Psoralens in Mycosis Fungoides[1]

Henry H. Roenigk, Jr.[2]

ABSTRACT—A 6-year follow-up of 12 patients with mycosis fungoides (MF) treated with psoralen photochemotherapy (PUVA) at the Cleveland Clinic revealed that 7 patients are clear of MF with 4 no longer receiving therapy and 3 on maintenance PUVA. Five patients died within 2 years of onset of therapy. Of 25 others with MF who were treated at Northwestern University, 20 are clear or partially clear after PUVA. Plaque-stage MF responds best to this type of treatment; Sézary syndrome, erythrodermic MF, and tumor-stage MF respond poorly to PUVA. — Natl Cancer Inst Monogr 66: 179–183, 1984.

As a form of malignant lymphoma that arises in the skin, MF over many years steadily progresses to plaques and tumors. Autopsy findings often reveal significant visceral involvement at the time of death (*1, 2*). The premycotic stage is characterized by scaly erythematous patches that clinically resemble nonspecific eczemas, psoriasis, or parapsoriasis. When skin lesions reach the plaque and tumor stage in the skin, the clinical and histologic diagnosis is more evident.

This distinct malignant cell lymphoma has its initial manifestations in the skin and is not a cutaneous manifestation of other lymphoma. The clinical course of MF is variable and may progress slowly through clinical stages over many years or evolve rapidly from cutaneous plaques and tumors to systemic diseases in a short time.

To choose the best therapy properly for each MF patient, a physician must do a complete work-up to evaluate potential systemic MF. The histopathologic diagnosis is often difficult even after multiple skin biopsies. The clinical features of mycosis fungoides often precede the histopathology.

Therapy of MF is controversial; its course varies from a long-term chronic process to early mortality. This variability in the natural course of the disease makes evaluation of therapy difficult. Staging of the disease (*3, 4*) is important for the physician; he must decide if therapy should be confined to skin lesions or be given for systemic effect to eradicate visceral involvement.

Van Scott and Kalmanson suggested the following classification of MF that has been modified slightly by the Scandinavian Mycosis Fungoides Study Group (*5*):

Stage	Clinical manifestations
I	Clinical pattern of MF unconfirmed histologically
II	Erythematous and infiltrated plaques of MF with typical MF histology
III	Tumors and ulcerations
IV	Lymph node involvement but no dermatopathic lymphadenopathy
V	Internal organ involvement with or without lymph node involvement (Sézary syndrome grouped with stage V because of consistent presence of typical lymphoid cells in blood and bone marrow)

The Mycosis Fungoides Cooperative Group in the United States has adopted a uniform staging system for MF (*6*). Useful information on 376 patients registered between November 1974 and December 1977 has been reported (*7*). The selection of patients for specific types of therapy depends on the stage of their disease. Patients in stages I and II will respond to many forms of therapy, but late stages IV and V usually require chemotherapy and even then the prognosis is not good.

Therapy of MF can be effective in stages I or II disease with topical steroids under occlusion (*8*). Haserick et al. (*9*) first reported remissions of MF lesions with topical applications of nitrogen mustard. The largest series and longest follow-up of MF patients have come from Van Scott and Kalmanson (*10*) and Vonderheid and associates (*11, 12*). Ten years of experience with 243 MF patients showed disease-free status in 53 and 38% of those with stages I and II disease, respectively. Relapse of MF may occur if maintenance therapy is discontinued even after a disease-free interval in excess of 3 years.

Trump and co-workers (*13*) first used high-energy electrons to treat MF in low-dose total skin radiation. Fuks and Bagshaw (*14*) and Fuks et al. (*15*) presented evidence that with up to 3,000 rad of 2.5-megavolt electrons, complete remissions occurred in 58% of the patients treated. Complete remission was observed in 80% of those with eczematous and limited plaque disease but only 30% in those presenting with tumors. Hoppe et al. (*16, 17*) have updated the Stanford electron beam studies in which doses greater than 3,000 rad were used. Five-year actuarial survival is 96% for limited plaque MF and 74% for generalized plaque, but it falls to 28% for tumors. They (*16, 17*) emphasize that long-term survival depends on initial extent of skin involvement.

Methoxsalen has been used for systemic treatment of vitiligo for over 20 years (*18*). Parrish et al. (*19*) reported successful treatment of psoriasis combining oral methox-

ABBREVIATIONS: MF = mycosis fungoides; UVA = UV radiation at 320–400 nm; PUVA = psoralen plus UVA; J = joule(s).

[1] Presented at the Conference on Photobiologic, Toxicologic, and Pharmacologic Aspects of Psoralens held in Research Triangle Park, North Carolina, March 1–3, 1982.

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salen given for systemic effect and high-intensity UVA (320–390 nm). Roenigk and Martin (*20*), Lynch et al. (*21*), and others (*22*) have confirmed the effectiveness of PUVA in the treatment of psoriasis and several other diseases.

The use of PUVA to treat MF was first reported by Gilchrest et al. (*22*) in 9 patients with various stages of the disease. I (*23*) had reported 12 patients with plaque, plaque plus tumor, and erythrodermic stages of MF who were treated successfully with PUVA and also correlated the histologic clearing of MF lesions with the apparent clinical clearing following PUVA.

For this present study, I reviewed a 6-year follow-up of the original 12 patients treated at the Cleveland Clinic (*23*) and expand the experience at Northwestern University with 25 additional patients with MF and 3 with parapsoriasis en plaque.

MATERIALS AND METHODS

Twelve patients at the Cleveland Clinic and 25 at Northwestern University who had clinical cutaneous lesions of MF were studied. The diagnosis was confirmed histologically by repeated skin biopsies. All biopsy specimens were reviewed and confirmed by members of the pathology panel of the MF cooperative study.

Complete evaluation of all patients included routine laboratory studies, microscopic examination of bone marrow biopsy specimens, roentgenograms of the upper and lower gastrointestinal tract, intravenous pyelograms, liver scans, brain scans, lymphangiograms, and lymph node biopsies. Only patients with no detectable visceral lesions of MF were entered into the PUVA therapy group.

Ten-milligram capsules of methoxsalen were given according to the method of Parrish et al. (*19*) based on patient body weight as follows: up to 50 kg, 20 mg; 51 to 65 kg, 30 mg; 66 to 80 kg, 40 mg; and over 80 kg, 50 mg. Most of the patients were taking 40 mg methoxsalen. Patients were instructed to take the medication 2 hours before their scheduled UVA therapy.

Phototherapy units used several systems with various high output UVA lamps (*24*). All lamps emit a continuous spectrum of long UV in the range of 320 to 400 nm with a peak emission between 350 and 380 nm. Initial exposure times of patients to high output UVA were based on the degree of pigmentation before therapy, history of ability to tan, and the type of phototherapy units to be used. Exposure times were increased with each treatment depending on patient response, evidence of erythema, and type of phototherapy unit used. Initial UVA dose was between 1.5 to 3 J/cm^2. Exposure times were increased at each treatment by at least 0.5 J/cm^2 or more depending on the presence of erythema. Light-blocking goggles were worn to protect the eyes from UV during therapy, and UVA-blocking sunglasses were worn before and after therapy. Photochemotherapy was usually given every 48 hours (three times/wk) during clearing.

Once the MF lesions had cleared completely, the patient was placed on a maintenance schedule. The UVA exposure during maintenance was the same as the patient's last treatment during the clearing phase. Maintenance was once per week for 4 weeks. If the patient remained clear, maintenance was decreased to once every 2, 3, or 4 weeks. All patients were encouraged to continue maintenance therapy with PUVA and not discontinue therapy as is done in treatment for psoriasis.

TABLE 1.—*Follow-up of 7 patients with MF given PUVA therapy at the Cleveland Clinic*[a]

Patient No.	Type of MF	Treatment status[b]	
		November 1980	November 1981
1	Plaque	M-1	M-4
2	"	M-4	"
3	"	Off PUVA	Off PUVA
4	Plaque and tumor	" "	" "
6	Plaque	" "	" "
7	Erythrodermic	M-2	M-2
9	Plaque	M-1	Off PUVA

[a] Treatment with PUVA began in 1975; all patients were clear at time of follow-up.

[b] M-1, M-2, and M-4 refer to maintenance once/wk, once every 2 wk, and once every 4 wk.

RESULTS

Cleveland Clinic Group

The specific details for the clearing of these patients has been reported (*23*). In 10 of the 12 patients, long-term follow-up of 3 years or greater was obtained (*25*).

Six-year follow-up (1975–81) of the original 12 treated at Cleveland Clinic revealed that 7 are clear of MF with 4 no longer receiving PUVA and 3 on maintenance therapy (table 1). Five patients died within 2 years after therapy began (table 2). Three had tumor stage lesions and failed to respond to electron beam and chemotherapy. One patient (#6) developed a squamous cell carcinoma on the arm that was adequately treated by excision. Histologic sections of skin from plaques of MF before and after PUVA confirmed the clinical clearing or lesions.

Northwestern University Group

An additional 25 patients with MF were treated from 1977 to 1981; a follow-up revealed 20 were clear or partially clear. Two received PUVA plus chemotherapy and 3 were given other therapy (table 3). Two patients with Sézary syndrome and 1 with erythrodermic MF died. The patients with parapsoriasis en plaque responded to PUVA, and those having plaque-stage MF responded the best (17 of 25 are clear); poor responses were shown by patients with tumor-stage MF. We found that those with dermatopathic lymphadenopathy responded as well as patients with no abnormal lymph nodes (table 4).

DISCUSSION

Initial studies with the use of PUVA to treat MF have demonstrated excellent effects in patients with eczematous, plaque, and early tumor stages of MF (*22, 23, 26*). Patients selected for PUVA showed no evidence of internal organ MF (stages I, II, III), and the histopathologic features of MF also cleared as did the clinical lesions (*23*). Although

TABLE 2.—*Follow-up of 5 patients with MF given PUVA and other therapy at the Cleveland Clinic*

Patient No.	Onset of PUVA therapy	Type of MF	Other therapy	Cause and yr of death	
				Cause	Yr
5	1975	Plaque and tumor	Electron beam, chemotherapy	MF	1976
8	1975	Plaque	Chemotherapy	Cardiovascular	1978
10	"	Plaque and tumor	"	MF	"
11	1976	Plaque	Electron beam, chemotherapy	"	"
12	"	Plaque and tumor	Electron beam	"	1979

the mechanism of action of PUVA against MF is not entirely clear, inhibition of DNA and some RNA synthesis occurs as well as a direct cytotoxic effect on abnormal lymphocytes in the dermis (*27*).

Long-term follow-up studies and larger series of patients with MF treated with PUVA are necessary before its true therapeutic value can be evaluated. Follow-up of my (*25*) initial 12 patients and now with another follow-up after 6 years show that 7 patients are clear of MF with 4 patients off PUVA and 3 on maintenance PUVA. Five patients died within 2 years of onset of therapy. Three of the patients had tumor-stage lesions and failed to respond to electron beam and chemotherapy. I have followed an additional 25 patients with MF and found it clear in 15 patients, partially clear in 2. The PUVA plus chemotherapy or other therapy was used in 6 patients and 2 died of MF. As a result of my experiences with the Cleveland Clinic and Northwestern University groups, I have concluded that PUVA is highly effective in eczematous, plaque- and early tumor-stage MF with remissions lasting up to 6 years. Maintenance PUVA therapy should be performed or relapses of MF will occur. Erythrodermic MF and Sézary syndrome have not responded as well to PUVA.

Warin (*28*) has recently summarized his experiences with 73 patients with MF who he studied up to 3 years. Of 54 patients with MF in stages I or II, 52 (98%) cleared clinically and histologically. Patients not kept on maintenance had frequent flares of this disease. Only 8 of 18 stage III patients cleared on MF, and the others required radiation and chemotherapy; several died. Warin concluded that PUVA was superior to topical nitrogen mustard. Other large studies have produced similar results (*5, 29–31*). Rotstein et al. (*32*) reported poor results from a series of 20 patients with MF. Examination of the data revealed many patients had advanced disease with lymph node and internal organ involvement. Those considered to have MF confined to the skin before treatment with PUVA began did well.

When used in patients with previous exposure to radiation therapy, arsenic, or chemotherapy, PUVA therapy may be co-carcinogenic (*33, 34*). Patients with MF are frequently exposed to these co-carcinogens before PUVA, and recent studies (*25, 35, 36*) have shown a high incidence of squamous cell carcinomas, basal cell carcinomas, and other epithelial tumors in these patients.

Because patients with stage III–IV disease have had little success with any of the above-mentioned treatment modalities, in the past few years physicians have tended to combine therapy. The following combinations with PUVA are being explored with early beneficial results:

Photochemotherapy and Nitrogen Mustard

DuVivier and Vollum (*37*) start patients on both photochemotherapy and nitrogen mustard simultaneously. Volden et al. (*38*) reported that PUVA will suppress the contact dermatitis caused by nitrogen mustard. Topical nitrogen mustard is used in long-term maintenance rather than PUVA.

Photochemotherapy Plus Retinoids

Oral aromatic retinoids (Ro10-9359) have been shown to augment the effect of PUVA and reduce the number of joules per square centimeter necessary to clear psoriasis (*39*). Patients with MF who have become resistant to PUVA therapy will become responsive again after receiving retinoids (*40*). Trials of combined therapy should be

TABLE 3.—*Follow-up of 25 patients with MF given PUVA therapy at Northwestern University*

No. of patients[a]	Clinical status
15	Clear
3	Clear[b]
3	[c]
2	Partially clear
1	Deceased, unknown cause
1	Deceased

[a] None had Sézary syndrome.
[b] Patients received PUVA and other chemotherapy.
[c] Patients received other therapy.

TABLE 4.—*Follow-up of patients with MF given PUVA therapy at Northwestern University*

Lymph node status	No. of patients:	
	Clear	Partially clear
Negative	1	3
Dermatopathic lymphadenopathy	7	1
Not investigated	5	1

initiated because of possible epithelial tumor suppression effect of retinoids.

Photochemotherapy Plus Chemotherapy

Although no official trials have been reported, personal experience has demonstrated that PUVA will help augment chemotherapy to reduce total tumor load and prolong survival.

Because of the good prognosis seen with electron-beam therapy, PUVA, and topical nitrogen mustard in early stages I and II MF, physicians have tended to delay combining these modalities with chemotherapy or other agents until the patient is in stage IV or V. Can topical whole-body therapy really cure MF and prevent spreading of the disease to other organs? Available data are conflicting. If topical whole-body treatment cannot, then more chemotherapy should be used at earlier stages. The immunosuppressive effect of these drugs in addition to other side effects have resulted in cautious use of early chemotherapy. Vonderheid (*41*) reviewed the philosophy of therapy of various stages of MF. Except for his choice of topical nitrogen mustard for early MF for which others (*25, 28, 42*) would use PUVA or electron beam (*17*), I would agree with his current plans for therapy. There is still room for new ideas and new approaches to the treatment of MF, especially for stages III–IV disease (*43–51*).

REFERENCES

(*1*) Epstein EH Jr, Levin DL, Croft JD Jr, et al: Mycosis fungoides. Survival, prognostic features, response to therapy and autopsy findings. Medicine 15:61, 1972

(*2*) Rappaport H, Thomas LB: Mycosis fungoides: The pathology of extracutaneous involvement. Cancer 34:1198, 1974

(*3*) Variakojis D, Rosas-Uribe A, Rappaport H: Mycosis fungoides—pathologic findings in staging laparotomies. Cancer 33:1589–1600, 1974

(*4*) Peojle JA, Winkelmann RK: Staging laparotomy in cutaneous T-cell disease. Arch Dermatol 117:543–546, 1981

(*5*) Molin L, Thomson K, Volden G, et al: Aspects of the treatment of mycosis fungoides. A report from the Scandinavian Mycosis Fungoides Study Group. Cutis 25:155–157, 160–161, 1980

(*6*) Griem ML, Moran EM, Ferguson DL, et al: Staging procedures in mycosis fungoides. Br J Dermatol 31(Suppl 2):362, 1975

(*7*) Green SB, Byar DP, Lamberg SI: Prognostic variable in mycosis fungoides. Cancer 47:2671–2677, 1981

(*8*) Farber EM, Zackheim HS, McClintock RP, et al: Treatment of mycosis fungoides with various strengths of fluocinolone acetonide cream. Arch Dermatol 97:165, 1968

(*9*) Haserick JR, Richardson JH, Grant DJ: Remission of lesions in mycosis fungoides following topical applications of nitrogen mustard. A case report. Cleve Clin Q 26:144–150, 1959

(*10*) Van Scott EJ, Kalmanson JD: Complete remissions of mycosis fungoides lymphoma induced by topical nitrogen mustard (HN2). Control of delayed hypersensitivity to HN2 by desensitization and induction of specific immunologic tolerance. Cancer 32:18–30, 1973

(*11*) Vonderheid EC, Van Scott EJ, Johnson WC, et al: Topical chemotherapy and immunotherapy of mycosis fungoides. Intermediate term results. Arch Dermatol 113:454–463, 1977

(*12*) Vonderheid EC, Van Scott EJ, Wallner PE, et al: A ten-year experience with topical mechlorethamine for mycosis fungoides. Cancer Treat Rep 63:681–689, 1979

(*13*) Trump JG, Wright KA, Evans WW, et al: High energy electrons for the treatment of extensive superficial malignant lesions. Am J Roentgenol Radium Ther Nucl Med 69:623, 1953

(*14*) Fuks ZY, Bagshaw M: Total-skin electron treatment of mycosis fungoides. Radiology 100:145, 1971

(*15*) Fuks ZY, Bagshaw MA, Farber EM: Prognostic signs and management of the mycosis fungoides. Cancer 32: 1385–1395, 1973

(*16*) Hoppe RT, Fuks ZY, Bagshaw MA: The rationale for curative radiotherapy in mycosis fungoides. Int J Radiat Oncol Biol 2:843, 1977

(*17*) Hoppe RT, Cox RS, Fuks ZY, et al: Electron-beam therapy for mycosis fungoides: The Stanford University experience. Cancer Treat Rep 63:691–700, 1979

(*18*) Fitzpatrick TB, Arnot KA, El Mofty AM, et al: Hydroquinone and psoralen in the therapy of hypermelanosis and vitiligo. Arch Dermatol 93:589–600, 1966

(*19*) Parrish JA, Fitzpatrick TB, Tanenbaum L, et al: Photochemotherapy of psoriasis with oral methoxsalen and longwave ultraviolet light. N Engl J Med 291:1207–1210, 1974

(*20*) Roenigk HH Jr, Martin MS: Photochemotherapy for psoriasis. Arch Dermatol 113:1667–1670, 1977

(*21*) Lynch WS, Martin JS, Roenigk HH Jr: Clinical results of photochemotherapy: The Cleveland Clinic experience. Cutis 20:37–41, 1977

(*22*) Gilchrest BA, Parrish JA, Tanenbaum L, et al: Oral methoxsalen photochemotherapy of mycosis fungoides. Cancer 38:683, 1976

(*23*) Roenigk HH Jr: Photochemotherapy for mycosis fungoides. Arch Dermatol 113:1047, 1977

(*24*) ———: Photochemotherapy for psoriasis—comparison of phototherapy units. *In* Psoriasis (Farber EM, Cox AJ, eds). New York: Yorke Medical Books, 1977, pp 464–466

(*25*) ———: Photochemotherapy for mycosis fungoides: Long-term follow-up study. Cancer Treat Rep 63:669–673, 1979

(*26*) Hodge L, Vella Briffa D, Warin AP, et al: Photochemotherapy in mycosis fungoides. Br Med J 2:1257, 1977

(*27*) Cripps DJ, Horowitz S, Hong R: Spectrum of ultraviolet radiation of human B- and T-lymphocyte viability. Clin Exp Dermatol 3:43, 1978

(*28*) Warin AP: Photochemotherapy in the treatment of psoriasis and mycosis fungoides. Clin Exp Dermatol 6:651–657, 1981

(*29*) Levi L, Cripps D, Galbiati G: Photochemotherapy for mycosis fungoides and parapsoriasis. Chron Dermatol 11:249–257, 1980

(*30*) Hofmann C, Burg G, Plewig G, et al: Photochemotherapy of cutaneous lymphoma: Oral and local 8-MOP–UVA treatment. Dtsch Med Wochenschr 102:675–679, 1977

(*31*) Lowe NJ, Cripps DJ, Dutton PA, et al: Photochemotherapy for mycosis fungoides. Arch Dermatol 115:50, 1979

(*32*) Rotstein H, Butler JM, Czarnecki DB, et al: The treatment of mycosis fungoides with PUVA. Australas J Dermatol 21:100–104, 1980

(*33*) Stern RS, Thibodeau LA, Kleinerman RA, et al: Risk of cutaneous carcinoma in patients treated with oral methoxsalen photochemotherapy for psoriasis. N Engl J Med 300:809, 1979

(*34*) Roenigk HH Jr, Caro WA: Skin cancer in the PUVA-48 cooperative study. J Am Acad Dermatol 4:319–324, 1981

(*35*) Abel EA, Deneau DG, Farber EM, et al: PUVA treatment of erythrodermic and plaque-type mycosis fungoides. J Am Acad Dermatol 4:423–429, 1981

(*36*) Verdick J: Squamous cell carcinoma: Occurrence in mycosis fungoides treated with psoralen plus long-wave ultraviolet radiation. Arch Dermatol 115:1338–1339, 1979

(*37*) DuVivier A, Vollum DI: Photochemotherapy and topical nitrogen mustard in the treatment of mycosis fungoides. Br J Dermatol 102:319–322, 1980

(*38*) Volden G, Molen L, Thomsen K: PUVA-induced suppression of contact sensitivity to mustine hydrochloride in mycosis fungoides. Br Med J 2:865–866, 1978

(*39*) Fritsch PO, Hönigsmann H, Jaschke E, et al: Augmentation of oral methoxsalen photochemotherapy with oral retinoic acid derivative. J Invest Dermatol 70:178–182, 1978

(*40*) Souteyrand P, Thivolet J, Fulton R: Treatment of parapsoriasis in plaques and mycosis fungoides with an oral aromatic retinoid (Ro10-9359). *In* Retinoids—Advances in Basic Research and Therapy (Orfanos CE, ed). Berlin: Springer-Verlag, 1981, pp 313–316

(*41*) Vonderheid EC: Evaluation and treatment of mycosis fungoides lymphoma. Int J Dermatol 19:182–188, 1980

(*42*) Spittle MF: Electron-beam therapy in England. Cancer Treat Rep 63:639–641, 1979

(*43*) Klein E, Holtermann O, Milgram H, et al: Immunotherapy for accessible tumors utilizing delayed hypersensitivity reactions and separated components of the immune system. Med Clin N Am 60:389, 1976

(*44*) Edelson RL, Brown JA, Grossman ME, et al: Antithymocyte globulin in treatment of T-cell lymphoma. Lancet 2:249, 1977

(*45*) Gould DJ, Rowell NF, Anthony H: Failure of antithymocyte globulin treatment in T-cell lymphoma. Lancet 2:1365–1366, 1977

(*46*) Edelson R, Faktor M, Andrews A, et al: Successful management of the Sézary syndrome. N Engl J Med 291:293, 1974

(*47*) Safai B, Reish L, Good RA: Failure of lymphopheresis in the treatment of Sézary syndrome and mycosis fungoides. Clin Res 26:576, 1978

(*48*) Zachariae H, Grunnet E, Ellegaard J, et al: Transfer factor as therapeutic agent in mycosis fungoides. Arch Dermatol 112:1324, 1976

(*49*) ———: Transfer factor as an additional therapeutic agent in mycosis fungoides. *In* Proceedings of the Third International Symposium on Transfer Factor (Kahn A, Kirkpatrick CH, Hill NO, eds). New York: Academic Press, 1979, pp 591–595

(*50*) Hayer PG, Kallor A, Michel B: Effect of levamisole in Sézary syndrome. Dermatologica 159:461–465, 1979

(*51*) Oseroff R: Monoclonal antibodies for mycosis fungoides. Presented at the 40th Annual Meeting, American Academy of Dermatology, San Francisco, California, December 7, 1981

Skin Cancer in Patients Treated With 8-Methoxypsoralen Plus Longwave Ultraviolet Radiation[1, 2]

Kenneth M. Halprin, Mary Comerford, *and* J. Richard Taylor[3]

ABSTRACT—Although severe biochemical and cytologic changes occur in both melanocytes and keratinocytes during psoralen photochemotherapy, a review of the published literature does not reveal any increased incidence of skin cancers from this type of therapy. Perhaps not enough time has elapsed to show such effects. — Natl Cancer Inst Monogr 66: 185–189, 1984.

Since PUVA therapy was first introduced in 1974, many studies have shown its efficacy for various skin diseases. The fear of skin cancers appears to be a main concern in the use of long-term PUVA therapy. A considerable amount of literature exists, and we would like to review that published on skin cancer and PUVA.

First of all, we found no reports of an increased incidence of leukemia or of melanoma, although 1 patient with leukemia and 1 with melanoma have been reported among those treated with PUVA, and this number would be expected by chance.

In regard to basal cell and squamous cell cancers, two questions need to be answered: 1) Does PUVA cause cancer primarily, i.e., is it a carcinogen? 2) Does PUVA act as a promoter? A great deal of information does exist on these questions. Data on 6 studies (*1–6*) are now available, and 1 study is to be published (Wolff K: Personal communication) involving approximately 5,000 patients followed for periods up to 6 years. Those who conducted this research agree that, in patients who have not had any previous carcinogenic exposure, PUVA during the first 6 years of use has not increased the incidence of skin cancer above that expected to occur from natural sunlight.

The answer to the second question is less clear. Patients who have had previous exposure to known carcinogens and are now being treated with PUVA are developing skin cancers at a rate of up to 2% per year. This may not be any higher than one would expect from the preceding carcinogen exposure alone.

What is a carcinogen? A carcinogen is a material which by itself will cause cancers to develop in an exposed population. Skin cancers caused by the classical inducers (UV light, arsenic, and ionizing radiation) characteristically do not appear immediately but only after a lag or latency of about 20 years. How is it determined whether a substance is a carcinogen? The material is applied or the procedure is performed by itself in the absence of other factors.

In regard to PUVA therapy, an investigator must compare the statistics on people who received it and those who have had no other exposure to carcinogens. Actually, another carcinogen is always present that cannot be removed: sunlight.

Using the 7 studies previously mentioned, one can then compare the detected cancers with the cancer rate found in a normal age-matched population whose cancers are due to sunlight (table 1).

In the Innsbruck study (*4*), 296 patients were treated who had no previous exposure to a carcinogen; no cancers were found. The expected incidence of 0.08/year should have produced 1 cancer. However, an incidence of 0.4% cancer cases/year, which was equal to that expected from sunlight alone, was reported by Roenigk and Caro (*3*). The same was true of the Stern et al. studies (*1, 2*).

Of 300 patients studied in Utah, only 2 had cancer (*5*); 1 was a patient who had a previous history of skin cancer, and the other had a neoplasm in a sun-exposed area of the body. In the European combined clinical trial (Wolff K: Personal communication), approximately 3,450 patients without prior carcinogen exposure took part; only 3 patients developed cancers.

Lassus and co-workers (*6*) treated 398 patients with psoriasis with PUVA for an average of 2 years; these patients had not been exposed previously to arsenic or ionizing radiation. One had a basal cell carcinoma of the scalp. This is the first reported study in which a control group of non-PUVA-treated patients with psoriasis were included. Three basal cell carcinomas developed in this group of non-carcinogen-treated patients.

From these latter studies, one can conclude that PUVA in the first 5 years of use is neither a carcinogen nor a promoter for skin cancers induced by sunlight.

Is PUVA a promoter? What is a promoter? A promoter is a material which by itself does not cause cancer. Instead, it serves to increase the numbers or decrease the latency when applied after a known carcinogen has acted. This is the indictment which has been made in some of the previous investigations, not that PUVA is a carcinogen but that it is a promoter acting on patients (who have had a

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ABBREVIATIONS: PUVA = 8-methoxypsoralen plus UV radiation at 320–400 nm; J = joules.

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[2] Supported in part by the Arthur O. and Gullan Wellman Foundation.

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TABLE 1.—*PUVA alone (with natural sunlight)*

Study	Investigator	No. of patients	Cancer incidence, %	
			Found	Expected
Innsbruck	Hönigsmann	246	0	0.08[a]
United States	Roenigk	520	0.4	0.5[a]
" "	Stern	851	0.5	0.5[a]
Utah	Wilson	300	0.4 (2)	?
Europe	Wolff	3,450	0.03 (3)	0.08[a]
Finland	Lassus	398	0.12 (1)	0.04[a]
				0.30[b]

[a] Value is that expected in the general population.
[b] Value is for 1,033 psoriasis patients who did not receive PUVA.

previous exposure to a carcinogen) to quicken the onset of their tumors or to increase the numbers of tumors above that which the carcinogen itself would have caused.

How is a promoter found? The rate of formation of new cancers in patients treated with the carcinogen alone is compared with the rate of formation of new cancers in patients treated with the carcinogen plus PUVA. Actually, in the studies just discussed, natural sunlight plus PUVA was compared with natural sunlight alone, and PUVA was not a promoter for natural sunlight. What about PUVA plus arsenic versus arsenic alone or PUVA plus ionizing radiation versus ionizing radiation alone? Only Lassus et al. (*6*) provided such information.

To emphasize the fact that patients who have been treated for many years with carcinogens do in fact get skin cancers, we reported data collected on a group of 29 such non-PUVA-treated patients over 5 years (*7*). The incidence was approximately 2% per year. Fourteen patients had cancers in the non-sun-exposed areas of the body, 13 of whom had squamous cell lesions.

Let us now turn to the published studies to see how high the cancer rates are in patients exposed to natural sunlight, a previous carcinogen, and PUVA (table 2).

Seventy-two of the 418 patients in the Innsbruck study had been exposed to arsenic, and all of the cancers (4 excluding 1 keratoacanthoma) developed in these patients (*4*). This gives an incidence of 2% per year in patients treated with sunlight, arsenic, and PUVA. In the Roenigk and Caro study (*3*), 25% of the patients had been exposed to ionizing radiation, and two-thirds of the skin cancers developed in these patients. The incidence rate for patients with cancers who had been treated with sunlight, ionizing radiation, and PUVA was 1.75% per year.

TABLE 2.—*PUVA plus carcinogen*

Study	Investigator	No. of patients	Cancer incidence/yr	
			No. of patients	Percent
Innsbruck	Hönigsmann	72	4	2
United States	Roenigk	170	6	1.75
" "	Stern	345	17	2.5
			36	2.5[a]
Europe	Wolff	1,150	18	0.4
Finland	Lassus	127	0	0.0

[a] This is the value if one assumes 50% of the cancers were in the group previously treated with ionizing radiation as was true of the 2-yr data.

Similarly, 25% of the patients Stern et al. (*1*) examined remembered exposure to ionizing radiation for their psoriasis, and two-thirds of the cancers were in this group. The calculated incidence of new cancer patients per year after sunlight, ionizing radiation, and PUVA was 2.5% per year at 2 years of follow-up. However, this figure should be reduced to 2% per year because 25% of these patients had had a cancer before receiving PUVA. It is well known that these patients will develop new cancers at a rate of 15% per year (*8*). The incidence rate did not change at the 4-year follow-up (*2*).

In the combined European study, 18 of approximately 1,150 patients estimated to have received a carcinogen previously developed skin cancer during an average follow-up of 20 months for an incidence figure of 0.4% per year. This is five times the expected rate in the absence of both PUVA and a carcinogen.

None of the 127 patients Lassus and co-workers (*6*) treated with PUVA for 2 years who had had previous arsenic therapy or ionizing radiation developed cancer. Of an estimated 200 patients with severe psoriasis who had received arsenic but had not been exposed to PUVA, 1 (0.5%) developed multiple basal cell carcinomas. The rates for skin cancer in non-PUVA-treated patients in the Finnish study were actually higher among both the carcinogen-treated and non-carcinogen-treated patients than in the PUVA groups.

What can we conclude from this review? We believe: 1) PUVA during the first 4 to 5 years of use has been shown *not* to increase the number of skin cancers expected on the basis of sunlight exposure alone. 2) Patients who have been exposed to sunlight, a carcinogen (arsenic or ionizing radiation, or both), and PUVA are developing cancers at a rate of up to 2% per year. This rate may not be higher than one would expect without PUVA. 3) As the use of arsenic and ionizing radiation in the treatment of psoriasis essentially stopped 15 to 20 years ago, the number of new patients presenting with such histories for PUVA therapy will decrease, which will lower the overall incidence of skin cancers in patients in PUVA programs. 4) If PUVA is a carcinogen, one might see a rise in the skin cancer rates in the 1990s when the latent period has passed. 5) Because of the long latency involved, it makes little sense to deny treatment to older patients. However, one should use more caution with younger patients.

REFERENCES

(*1*) STERN RS, THIBODEAU LA, KLEINERMAN RA, et al: Risk of cutaneous carcinoma in patients treated with oral methoxsalen photochemotherapy for psoriasis. N Engl J Med 300:809–813, 1979

(*2*) STERN RS, PARRISH JA, BLEICH HL, et al: PUVA (psoralen and ultraviolet A) and squamous cell carcinoma in patients with psoriasis. J Invest Dermatol 76:311, 1981

(*3*) ROENIGK HH JR, CARO WA: Skin cancer in the PUVA-48 cooperative study. J Am Acad Dermatol 4:319–324, 1981

(*4*) HÖNIGSMANN H, WOLFF K, GSCHNAIT F, et al: Keratoses

and nonmelanoma skin tumors in long-term photochemotherapy. J Am Acad Dermatol 3:406–414, 1980
(5) WILSON RG: Community PUVA center—four-year experience. Dermatology 3:29–33, 1980
(6) LASSUS A, REUNALA T, IDÄNPÄÄ-HEIKKILÄ J, et al: PUVA treatment and skin cancer: A follow-up study. Acta Derm Venereol (Stockh) 61:141–145, 1981
(7) HALPRIN KM: Psoriasis, skin cancer and PUVA. J Am Acad Dermatol 2:432–433, 1980
(8) BERGSTRESSER PR, HALPRIN KM: Multiple sequential skin cancers. The risk of skin cancer in patients with previous skin cancer. Arch Dermatol 111:995–996, 1975

DISCUSSION

R. Stern: With respect to risk–benefit analysis and with PUVA as it now stands in the therapy of psoriasis, I would agree with Dr. Halprin's recommendations. However, I disagree with him in his interpretation of certain aspects of my data, which suggest a strong relationship between cumulative dose of PUVA and the development of squamous cell carcinoma. Although most marked in patients previously treated with ionizing radiation, the increased risk seen in high-dose patients is not confined to this group.

I would like to discuss 4 of the studies that Dr. Halprin reviewed and point out one important difference in the studies as summarized.

In addition to the ones that Dr. Halprin pointed out, I think the principle differences among these studies that must be considered are the mean PUVA dose and the duration of follow-up. As of 1981, the 16-center cohort's mean dose was greater than 1,400 J. The mean PUVA dose used in the Finnish study at the time of its publication was less than 350 J.

Dr. Hönigsmann's group used about one-half the dose we did at the time they published their results, but Dr. Roenigk did not explicitly calculate the dose. When talking to Dr. Roenigk, I discovered their doses were certainly substantially (if not by an order of magnitude certainly by multiples) smaller than the doses our group used. This is an appreciable difference in exposure. The duration of follow-up in the Finnish study was much shorter than ours, but, in fact, their completeness in examination was quite exemplary.

If the development of squamous cell carcinoma is occurring independent of exposure to PUVA, we would expect that the ratio of squamous cell carcinoma to basal cell carcinoma should be identical for patients with given risk characteristics in each of the three PUVA dose ranges. This is clearly not the case.

Because I believe the effect of PUVA is principally related to squamous cell carcinoma (a relatively infrequent tumor if you look at aggregate risk rates which include basal cell carcinoma) you essentially understate its effect on the risk of squamous cell carcinoma tumors because not much change occurs in the more frequent type of tumors in the general population, i.e., the basal cell tumors.

If you look at the data for our low-risk group and you consider tumor rates in the aggregate, I agree, little change is noted in patients not exposed to ionizing radiation, substantial doses of UV at 290–320 nm, or tar. However, if you separate the patients with squamous cell carcinoma and look at the expected rate for this carcinoma, you will note an approximate fivefold increase in the risk of squamous cell carcinoma in this group lacking substantial exposures to carcinogens other than PUVA. There is no significant increase in basal cell carcinoma among these people. Although the risk of increase for all tumors in this group is small, that for squamous cell carcinoma is substantial.

Also, because the risk group was ascertained before the first PUVA treatment, if no relationship exists between PUVA and the development of squamous cell carcinoma, I would expect that the ratio of squamous cell carcinoma to basal cell carcinoma should not vary in a given risk group. In other words, if PUVA is unrelated, why should it not be essentially a 2:3 ratio in both exposure groups? I think that until we can explain that finding, we have strong reason to believe that prolonged exposure to high doses of PUVA (i.e., at least 1,000 J) increases the patients' risks for skin tumors.

I do not know the mechanism of that effect, and I agree that it is unlikely that what we have observed is solely a primary carcinogenic effect of PUVA. One reason that I am anxious to continue our study and our surveillance is to determine, in 5 or 10 more years, whether we will see a substantial increase in the number of tumors appearing in the low-risk population. It is also important that we continue to monitor this population for lymphoma and melanoma as well as other systemic neoplasms.

Although I cannot tell you anything from my data about the mechanism of action, I think the strong statistical relationships we see are hard to explain in any way except to believe that something occurs in people who receive high doses of PUVA that increases their risk of developing squamous cell carcinoma.

I think caution demands that we pay attention to this observation while we attempt to determine the ultimate mechanism. Up to this point, I think the clinical significance of these observations is to make us concerned enough to support the judicious use of PUVA and to increase the need for further study. These observations are not a contraindication to the use of this therapy. As Dr. Wolff noted, the risk-to-benefit ratio for the individual patient and the therapeutic alternatives open to that patient should determine the therapy chosen.

K. M. Halprin: I think this is another way of getting around the problem of not having a control group. Is there a dose–response relationship? If these data are valid (and I hope that Dr. Stern will continue with his study because it is the best one now being conducted), and these figures confirm that a dose–response relationship does exist even in the low-risk people who are not exposed to carcinogens, then we will have positive evidence of promotion at least.

More data on promotion are needed, and no one has seen statistics on this aspect. Other centers have not repeated work on the dose–response relationship. I think more time is needed to see how these figures work out. This is the first presentation of the dose–response relationship even in a low-dose group. These are data for which we should be looking.

Stern: My statement was based on the original journal article and indicates no significant increase in pre-PUVA tumor in this group.

R. Brickl: What you said I think will hold true, but only if this division of, let us say, low and high numbers of PUVA treatments is the only thing which separates this group.

Could it not be, for instance, that this high number of PUVA treatments indicates that the patients have a much more severe psoriasis or that it is psoriasis that seems to come back more frequently? This perhaps could be a sign that these people have also been treated before with much more frequency either with X-rays, arsenic, or similar therapy. I think we really have to pursue this further before we can argue the point.

Stern: May I respond to that? Dr. John Melski and I have been interested in studying the determinants of long-term use of PUVA therapy from two perspectives: 1) the continuation of PUVA therapy in the long term, and 2) the utilization and factors that predict or are at least associated with long-term utilization (among those patients who continue to rely on the therapy).

In fact, patients with the most severe psoriasis in our group were most likely to drop out of therapy, and, therefore, if you look at patients treated with ionizing radiation who also have severe psoriasis, they, on the average, tend to have less PUVA exposure than those without exposure to ionizing radiation. Also, older patients were more likely to be exposed to ionizing radiation and to have used less PUVA. In this country, it is largely for insurance reasons. However, if you look at the patients with characteristics that, independent of PUVA, are associated with skin cancer risk and then examine the long-term PUVA use independent of development of tumors, patients with these risk factors not only use less PUVA treatment but are less likely to stay on it for the long term than those lacking those risk factors.

T. B. Fitzpatrick: Dr. Stern, how do you explain the fact that, since 1974 we have had 2 studies going, 1 in Boston and 1 in Vienna? The Boston studies are farther ahead of those in Innsbruck because we started about 1 year earlier. If you go back and take the same cumulative joules that Dr. Wolff had, do you have the same incidence of cancer as in his population?

Stern: I do not know. In our first year of study when most patients were still using PUVA and the average was about 40 to 50 treatments/patient, which should be about 500–600 J, we saw some tumors, many of which I thought were probably preexisting.

In this first year, we saw almost no squamous cell carcinomas. I think we must have done something to our group because of the way we administered PUVA in the mid-1970s (with higher intensity of therapy, greater degree of phototoxicity, high cumulative dose, or other factors) that set up our population for an increase in skin cancer risk. I cannot even speculate about mechanisms of action.

As Dr. Halprin pointed out, we did have a population with lots of exposure to other potential carcinogens before the PUVA therapy. I was interested to hear that some of the other groups that he reported on had almost as high a percentage of patients with similar kinds of exposures. So perhaps our patients are not as unique as we surmised as to how high a risk group they are, i.e., pre-PUVA, based on exposures. Our group is certainly unique in the way we used PUVA during those first 2 to 3 years and in our current cumulative doses which are two to five times those of other groups.

Halprin: The squamous cell-to-basal cell ratio does not bother me because that happens with all carcinogens. Of the 29 patients we reported who developed skin cancer in the *absence* of PUVA treatment, 14 developed squamous cell carcinomas and 13 cancers were in non-sun-exposed areas. Five of the cancers were perianal: 1 was in the gluteal cleft, 2 were perianal, and 2 were on the scrotum; all 5 were probably tar induced.

M. Pathak: I do not know why PUVA-treated patients develop these squamous cell tumors. I see patients developing them in the lower extremities.

Halprin: You see the same thing without PUVA. The patients I have reported have never received it. They received arsenic, X-ray therapy, tar, and UV light. For those who developed perianal tumors, you can presume the tar is the most likely causative agent, but regardless of the carcinogen, they all develop squamous cell carcinomas in the non-sun-exposed areas.

K. Wolff: The difference in the total cumulative doses of UVA between the American and European studies is the result of different approaches to treatment and does not reflect differences in the duration of treatment or follow-up. For instance, our study, which was referred to as the Innsbruck study, dates back to the summer of 1974. The differences of total joules is tremendous. Also, another factor is involved. More than 60% of our patients have skin type III, but we could not find a correlation between the skin types and the number of tumors; the number of tumors needed to perform such correlations was simply not high enough. How this will go in the future I do not know. We have followed these patients carefully, and these data are now 1½ years old; still, we have seen no further increase in tumors so far.

J. H. Epstein: Dr. Halprin mentioned that carcinogens induce squamous cell carcinomas, but that depends on where the cancer occurs. If it occurs on the head or neck, it is usually a basal cell carcinoma.

Halprin: Yes, it usually is a basal cell carcinoma.

Epstein: Is that because of ionizing radiation, UV radiation, or the like?

Halprin: Yes.

D. M. Carter: We should re-emphasize differences in methodology in these 2 studies. I remember well the requirement for following a predetermined schedule of treatment, assigned at random on entry into the American study. This was not the procedure used in the European series, and even from the start, there was more individualization in Europe.

In the United States study, we were "honor bound" to randomize. Some of our patients were clearly on a schedule that provided too much or not enough therapy, but we tried to continue with it to collect the data. The populations were not the same because of the differences in individualization of treatment.

Fitzpatrick: Actually, we will never work this out

because if something is done wrong in the beginning, you do not have another population to follow, so we will never resolve the problem.

Halprin: Unfortunately, we are struck with this group of severely affected patients who have been badly treated and are going to show us off in the worst light forever because we lack a young population not treated this way with whom we can start.

Fitzpatrick: Except in Europe we do.

Stern: Do you not think it is better to know the upper limits of the toxicity of an agent rather than its lower limits?

Halprin: Yes, of course.

Chronic Cutaneous Effects of Long-term Psoralen and Ultraviolet Radiation Therapy in Patients With Vitiligo[1]

Terence J. Harrist,[2] Madhu A. Pathak,[3] David B. Mosher,[3] *and* Thomas B. Fitzpatrick[3]

ABSTRACT—Vitiligo is refractory to most therapeutic modalities. To assess the efficacy of a variety of PUVA therapies, we enrolled 596 subjects in a prospective study, and 230 were followed for up to 55 months. Various psoralen derivatives and dosage schedules were used. Each subject was examined at yearly intervals for therapeutic response and evidence of chronic PUVA toxicity. At 4 years after therapeutic inception, 29 (13%) developed lesions in remaining vitiliginous macules. Clinically, hyperkeratotic macules and hyperkeratotic, lichenoid, and telangiectatic papules were discerned. Histologic examination of these lesions revealed them to be actinic and lichenoid keratoses, verruca vulgaris, and hyperkeratosis with either hyperplasia or atrophy. No tumors were present. In perilesional skin, dermal collagen and elastic tissue degeneration, much greater in degree than reported in psoriatic skin, was observed. In this group of PUVA-treated patients, no increased risk of carcinoma was apparent during the follow-up period. — Natl Cancer Inst Monogr 66: 191–196, 1984.

Vitiligo is an idiopathic acquired amelanoderma characterized by evolving chalk-white macules. Circumscribed amelanosis results from a loss of functional epidermal and follicular melanocytes. This disorder affects 1 to 3% of the world's population with equal sex predilection (*1, 2*). A few patients find their exposed vitiligo macules to be particularly susceptible to the acute (sunburn) and chronic effects of UV radiation. Treatment of vitiligo has included many modalities but photochemotherapy (PUVA) with oral psoralens (PUVA or PUVA and UV solar radiation) plus UVA (320–400 nm) has yielded the most successful repigmentation. Although the cutaneous toxicity of PUVA therapy of psoriasis has been much discussed (*3–7*), scant mention has been made of the chronic toxicity of PUVA therapy of vitiligo, which is the subject of our report.

MATERIALS AND METHODS

Of 596 East Indian patients originally enrolled, 230 patients with vitiligo were followed up to 55 months. Each patient was examined by at least 2 physicians and checked annually for PUVA-induced pigmentary changes, phototoxicity, and other changes associated with chronic effects of sunlight exposure. Patients were treated with sunlight plus either orally administered 0.3–0.6 mg 8-MOP/kg, 0.6–3.6 mg TMP/kg, 0.4–0.8 mg psoralen/kg, or a combination therapy of 0.3 mg 8-MOP/kg and 0.6 mg TMP/kg. The various dosage schedules are given in table 1. Patients were treated every other day up to 55 months and interviewed every 2 months; they underwent yearly assessment of their repigmentation with particular attention given to signs and symptoms of cutaneous and systemic phototoxicity as well as other skin changes. At the end of 4 years of study, each of the 230 patients was evaluated by least 3 physicians. Twenty-nine patients had developed discrete lesions in amelanotic areas. From 10 representative subjects with the most severe changes, 26 lesions and surrounding nonpigmented vitiliginous macules were biopsied by use of the standard 4-mm Keyes punch biopsy technique and 1% Xylocaine. One patient had 10 lesions biopsied, and the other 9 patients, 1 to 3 lesions each. Specimens were formalin-fixed, paraffin-embedded, sectioned at 4-μ thickness, and stained with H & E. Some specimens were stained with Congo red and Masson-Fontana techniques and examined.

The following grading system was used for evaluation of histologic changes:

Grade	Histologic alterations
0	No change
±	Trace, focal
1+	Trace, diffuse
2+	Moderate, focal
3+	Moderate, diffuse
4+	Severe, extensive

The quantity of infiltrating cells was semiquantitatively graded as follows:

Grade	Infiltrate
0	None
1+	1–5 Inflammatory cells/venule in the superficial plexus
2+	5–10 Inflammatory cells/venule in the superficial plexus
3+	Patchy bandlike infiltrate
4+	Confluent bandlike infiltrate

ABBREVIATIONS: PUVA = psoralen plus UV radiation at 320–400 nm; 8-MOP = 8-methoxypsoralen; TMP = 4,5′,8-trimethylpsoralen; H & E = hematoxylin and eosin.

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RESULTS

Clinical Assessment

Of the 230 patients examined, 29 had lesions which were classified into 4 types: hyperkeratotic macules and hyperkeratotic, lichenoid, and telangiectatic papules. All lesions were less than 1 cm in diameter and most papules did not exceed 5 mm in diameter. All lesions were confined to remaining vitiliginous macules. No lesions arose in uninvolved normal or repigmented skin. None of the observed lesions had been present at the previous yearly evaluation.

In 24 patients, the lesions were located on the lower legs, usually on the anterior surface or the ankles. Of the remaining 5 patients, 3 had 1 lesion each on the lip, face, or neck; another had 5 lesions on the chest; and the last had 6 lesions on the back. Nine patients had a single lesion, 14 had 2 to 6 lesions, and 6 had more than 6 lesions each. Two patients in the latter group had lesions of all 4 clinical types.

These lesions developed in all treatment groups (table 1). Thirty-two percent of the patients on 3.6 mg TMP/kg and 23% of those on 0.3 mg 8-MOP/kg developed lesions. Several clinical types were observed in most groups, and all types of lesions were observed in the 3 patients receiving TMP alone. No lesions were observed in the group receiving 0.3 mg psoralen/kg.

Those who developed lesions were older than the group average (32 yr), had vitiligo longer, had longer duration of therapy, and better overall repigmentation response than the group as a whole. Of the 29 patients with lesions, 8 were female and 21 were male. They ranged from 18 to 62 years of age (mean, 41 yr). Those with lesions had had vitiligo on the average 9–10 years longer than the group as a whole. The average duration of PUVA therapy was 40 months with a 30- to 55-month range as compared with 35 months of therapy with a 22- to 55-month range for the entire group. Of all patients treated over 48 months, 17 had developed lesions. No lesions developed in patients with less than 30 months of therapy. About 55% of this group with lesions had achieved over 75% total repigmentation.

Although precise treatment histories were difficult for us to elicit because of the numerous folk medicine remedies for vitiligo in India, we ascertained that most of the patients had previous therapy with topical psoralens and sunlight [usually unsubstituted psoralen 1% ointment or herbal paste made from "Bavachee" or *Psoralea corylofolia* (Leguminosae) that contains psoralen and other furocoumarins].

Histologic Assessment

Table 2 lists the histologic features of the 26 lesions examined and biopsied: 6 were papillary epidermal hyperplasia; 5, actinic keratosis; 5, epidermal hyperplasia with hyperkeratosis; 4, lichenoid keratosis; 3, verrucae vulgaris; and 3, epidermal atrophy with hyperkeratosis.

The lesions diagnosed as papillary epidermal hyperplasia (fig. 1) had moderate to marked hyperkeratosis, scant to moderate hypergranulosis, mild to marked acanthosis, and papillomatosis. Scant apoptosis was observed. A slight focal perivascular lymphocytic infiltrate was present. Vascular changes were minimal. Papillary dermal sclerosis was mild in association with focal degenerative collagen alteration.

Those found to have epidermal hyperplasia with hyperkeratosis corresponded to the keratotic papules observed grossly. Histologically, they were distinguished by less acanthosis and papillomatosis. Other keratotic macules were epidermal atrophy with striking hyperkeratosis (fig. 2). The 5 keratotic papules diagnosed as actinic keratosis (fig. 3) had only mild cytologic atypia.

Four additional keratotic papules were lichenoid keratoses (fig. 4) with moderate focal acanthosis and scant papillomatosis. Vacuolization of the basal layer was moderate to marked. A marked band-like lymphocytic infiltrate with few histiocytes was observed. Moderate focal endothelial hypertrophy and mild endothelial degenerative

TABLE 1.—*Vitiligo project histology study*

Assigned group code	Therapy		No. of patients evaluated	No. of patients with lesions	Percent with lesions	Types of lesions			
	Drug	Amount, mg/kg				Keratotic macule	Keratotic papule	Lichenoid papule	Telangiectatic papule
A	8-MOP[a]	0.3	34	4[b]	12		1	1	
	TMP	0.6							
B	"	0.8	22	3[c]	14	2	2	1	2
C	8-MOP	0.3	26	6	23	3		1	
D	TMP[a]	0.8	29	3	10	1	1	1	2
E	8-MOP	0.6	23	2	9	2			
H	TMP	3.6	22	7	32	5	2	1	2
½P	Psoralen	0.3[d]	37	0	0				
P	Psoralen	0.6	21	2	10	2	2		
PP	"	1.2	16	2	12		2	2	
Total			230	29	13	15	10	7	6

[a] This group had placebos for the first 12 mo.
[b] We have no data for 2 patients with respect to lesion(s).
[c] We have no data for 1 patient with respect to lesion(s).
[d] This group had a 24-mo follow-up.

TABLE 2.—*Histologic changes in lesions in patients with vitiligo*

Histological features	No. of lesions with histologic diagnosis[a]					
	Papillary epidermal hyperplasia	Epidermal hyperplasia with hyperkeratosis	Verruca vulgaris	Lichenoid keratosis	Actinic keratosis	Epidermal atrophy with hyperkeratosis
	6	5	3	4	5	3
	Grading					
Epidermal						
Hyperkeratosis	2–4+	1–4+	3–4+	2–3+	3–4+	3–4+
Hypergranulosis	±–2+	0–2+	2–4+	0–2+	0–2+	1+
Parakeratosis	0–1+	0–2+	1+	2+	2–3+	+/−
Acanthosis	1–3+	0–1+	2–3+	1–2+	1–3+	0
Papillarity	1–4+	0–1+	4+	±–+	0–2+	0
Atypia	0	0	0	0	±–1	0
Apoptosis	±	±–2+	0–±	2	0–3+	+/−
Basal vacuolization	0	0–+	0	2–3+	0	1+
Basement membrane zone thickening	0	0	0	0	0	
Melanocytes	0	0	0	0	0	0
Papillary dermis						
Sclerosis	2–3+	1–4+	2+	2+	2–3+	2–3+
Infiltrate	Perivascular	Perivascular	Perivascular	Bandlike	Perivascular	−
Lymphocytes	1+	0+	0	3+	±–3+	+/−
Histiocytes	0	0	0	+/−	0–+/−	0
Amyloid bodies	0–1+	0–1+	0–±	±–2+	0–+/−	+/−

[a] All lesions had the clinical appearance of a keratotic papule; in addition, papillary epidermal hyperplasia and epidermal hyperplasia with hyperkeratosis also showed some lichenoid papule characteristics. Grading: 0 = no change; ± = scant; +/− = some/none; 1 = mild; 2 = moderate; 3 = high: < marked but > moderate; 4 = marked.

changes were observed. Moderate focal collagen degeneration extended into the reticular dermis.

A few colloid–amyloid bodies, as shown by Congo red stain, were seen in lesions of all treatment groups, but solar elastosis was not observed.

None of the lesions were basal cell carcinoma, squamous cell carcinoma, or malignant melanoma.

Perilesional Vitiliginous Skin

Around the discrete lesions, epidermal alterations were less striking and the dermal changes were similar qualitatively and quantitatively to that of lesional skin, regardless of lesion type. In the perilesional skin examined, hyperkeratosis and hypergranulosis were uniformly manifest. Epidermal maturation disorder in each of the 10 subjects was slight in 8 and moderate in 2. Apoptosis varied from 0 to 2/linear mm of epidermis. No melanocytes were identified by Fontana-Masson or H & E staining in any biopsy specimen.

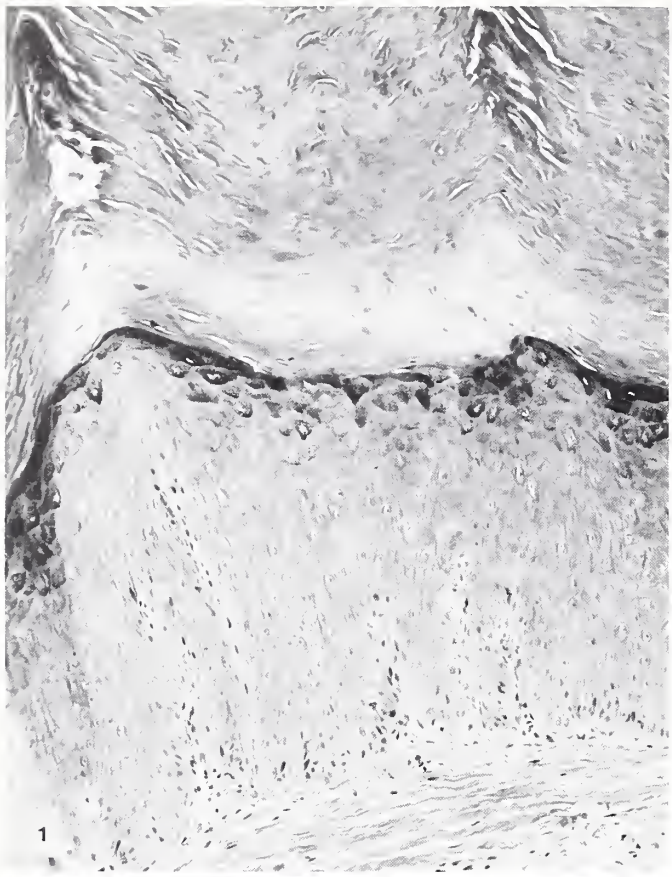
The dermal changes were striking including marked subepidermal hyalinization, abnormal arrangement, and size of collagen bundles in every biopsy specimen; the extent of the latter was slight in 5, moderate in 3, and marked in 2. Each specimen contained colloid bodies varying from 0 to 2/linear mm. Only rare colloid–amyloid bodies were identified. Striking loss of subepidermal elastic tissue with coarsening and fragmentation of the deeper elastic tissue was evident in each specimen. In most, it was apparent to the depth of the biopsy specimen, which varied from 3 to 5 mm. Only slight endothelial activation was generally observed.

DISCUSSION AND CONCLUSIONS

Discrete pathologic lesions developed in nonresponsive amelanotic macules in 13% of 230 patients with vitiligo treated up to 55 months with sunlight plus either 8-MOP, TMP, TMP and 8-MOP, or psoralen. The absence of lesions on clinically normal or repigmented skin reflects the photoprotective property of melanin. All lesions developed during the third and fourth years of uninterrupted therapy. Because lesions developed in all treatment groups, one cannot conclude that lesions are likely to develop with a particular psoralen derivative. In general, those patients who had received TMP developed more lesions than those who received 8-MOP or psoralen.

Four types of lesions were observed clinically: keratotic macules on an erythematous base, hyperkeratotic papules often on a larger erythematous macule, nonkeratotic white telangiectatic papules, and lichenoid keratotic papules which had a slightly raised keratotic rim (porokeratosis-like). None of the lesions were pigmented; all were distinct and nonconfluent. Most patients had but 1 or 2 types of lesions.

The fact that most lesions were located on the lower legs may be explained at least partially. Because many patients had prior sun exposure and various indigenous remedies



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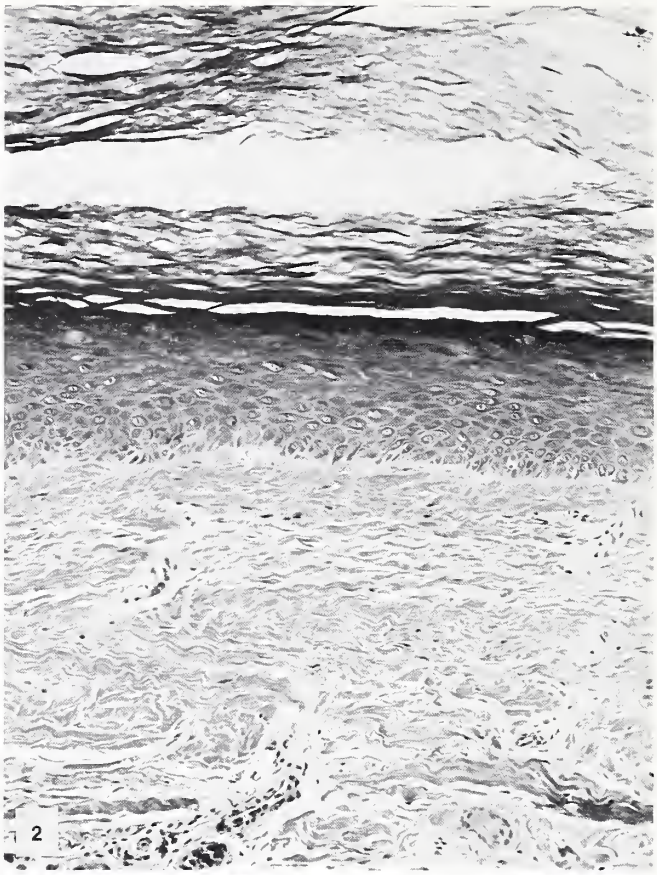

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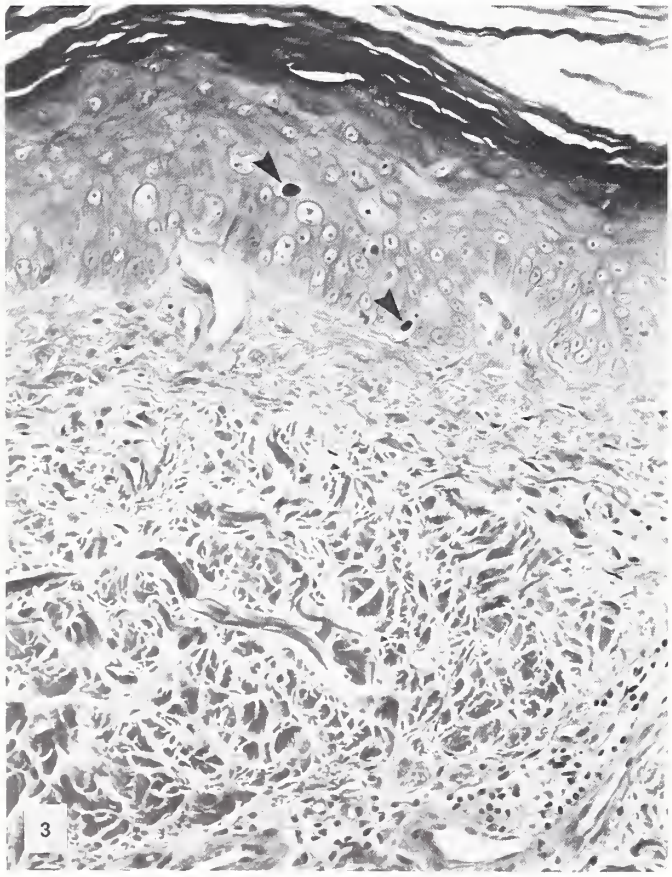

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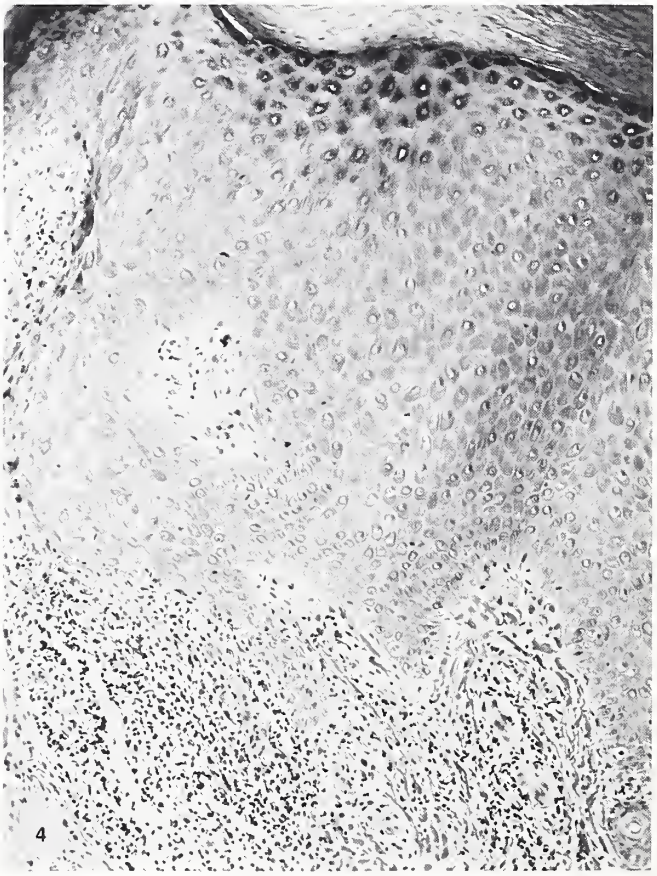

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for their vitiligo, isolation of a single pathogenesis of these lesions is probably impossible. All postulates presume that sunlight exposure as well as psoralen therapy is important to the pathogenesis of these lesions. In some patients, the lower legs often responded poorly even in those whose skin repigmented well elsewhere. Such individuals were often highly motivated to continue treatments and thereby accumulated large amounts of sun exposure or UVA doses to these amelanotic areas. However, in other treatment-resistant areas, the dorsal hands and digits (areas which also sustain significant amounts of UV light), only few lesions were observed. Therefore, factors other than solar radiation may predispose the legs and ankles.

The roles of PUVA and moderate irradiation (290–320 nm) cannot be clearly segregated. The occurrence of leg lesions in psoriatics treated with PUVA suggests that PUVA must be an integral part of the pathogenesis, alone or as a promoter (*3*). Nevertheless, the benign appearance of lesions biopsied and only mild focal atypia in the 5 actinic keratoses observed, particularly in face of the long duration of uninterrupted therapy, is reassuring as is the absence of frank skin cancer.

A good clinicopathologic correlation was made between clinical and histopathologic observations (table 2). The keratotic macules were actinic keratoses or epidermal atrophy with hyperkeratosis. The lichenoid papules were primarily lichen planus-like keratoses or lichenoid actinic keratoses. In those lichenoid papules with papillary epidermal hyperplasia, the hyperkeratosis filled the spaces between the papillae to produce a flat surface. Keratotic papules exhibited epidermal hyperplasia and hyperkeratosis. Telangiectatic papules were characterized by epidermal atrophy and hyperkeratosis.

In the 1 patient with 10 lesions biopsied, verrucae vulgaris were identified as well as papillary epidermal hyperplasia without the classical features of verrucae, such as vacuolated granular cells, abnormal keratohyaline granules, or nuclear inclusions. However, these may represent older verrucae vulgaris which may not demonstrate these findings or contain viral particles. Therefore, all lesions in this patient may represent verrucae in various stages of evolution. It is possible that this patient was more susceptible to primary cutaneous viral infection or to reactivation of latent infection because of alterations in lymphocyte function secondary to psoralen photochemotherapy. Incidental development of these lesions cannot be totally excluded.

The relationship of actinic damage to the development of solar keratosis is well known. Lichen planus-like keratoses most frequently occur in sun-exposed skin (*8*). In the observed actinic keratoses, the cytologic atypia was not marked nor was downward budding prominent.

Small numbers of apoptotic cells were found in all biopsy specimens. These have been found in normal skin and are present in increased numbers in lupus erythematosus, lichen planus, and photodermatoses (*9*). Except in lichen planus-like keratoses, no correlation between the number of intraepidermal apoptotic cells and dermal colloid bodies was demonstrable. A few dermal colloid bodies exhibited green birefringence under polarized light after being stained with Congo red. Amyloid deposits, probably originating from epidermal apoptotic cells (*10*), have previously been demonstrated in the dermis of PUVA-treated patients (*11–13*).

The hyperkeratosis and epidermal maturation disorder are similar in degree to those noted in psoriatic patients treated with PUVA (*13, 14*). However, the degree of apoptosis is generally greater. This variable is dependent on the frequency of PUVA therapy and the time of the last treatment.

Other changes, i.e., papillary dermal fibrosis and the markedly reduced number of reticular dermal fibroblasts and collagen fragmentation in our patients, also indicate a protective function for melanin in reducing damage to dermal structures. Although Gschnait et al. (*14*) and others have reported loss of superficial elastic fibers and subepidermal sclerosis in psoriatics, no comment has been made concerning deep collagen or elastic tissue abnormalities noted in nonmelanized skin of patients with vitiligo. The subepidermal hyalinization and elastic tissue degeneration we noted, as well as the changes in collagen, are much more striking than those noted in PUVA-treated psoriatics (*13, 14*). The number of colloid–amyloid bodies and their frequency per linear millimeter is not known in PUVA-treated psoriatics. However, the frequency appears to be greater in non-repigmented skin of patients with vitiligo than in our previous experience with psoriatic patients (*8*).

Thus no evidence for increased risk for cutaneous carcinoma or melanoma was apparent in this group of patients. However, lichenoid keratoses were unexpectedly frequent. Unfortunately, no age-matched control population was available. Melanin clearly is protective because no lesions were observed in normally melanized or re-melanized skin.

REFERENCES

(*1*) Mosher DB, Fitzpatrick TB, Ortonne JP: Abnormalities of pigmentation. *In* Dermatology in General Medicine (Fitzpatrick TB, Eisen AZ, Friedberg IN, et al, eds), 2d ed. New York: McGraw-Hill, 1979, pp 568–629

(*2*) Mosher DB, Pathak MA, Fitzpatrick TB: Vitiligo: Etiology, pathogenesis, diagnosis and treatment. *In* Update: Dermatology in General Medicine (Fitzpatrick TB,

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FIGURE 1.—Epidermis exhibits psoriasiform and papillary hyperplasia. Dermis is markedly abnormal with lamellar dermal fibrosis, which was noted in perilesional skin as well. H & E. × 160

FIGURE 2.—Loss of rete ridges and marked hyperkeratosis is present. *Note* abnormal arrangement and size of collagen bundles. H & E. × 160

FIGURE 3.—Slight keratinocytic atypia and apoptosis (*arrowheads*) are noted. H & E. × 256

FIGURE 4.—Irregular epidermal hyperplasia in which wedgelike hypergranulosis and "saw-tooth" rete are observed. A bandlike lymphocytic infiltrate focally obscures the dermo-epidermal junction. H & E. × 160

Eisen AZ, Wolff K, et al, eds). New York: McGraw-Hill, 1983, pp 205–225

(*3*) Stern RS, Thibodeau LA, Kleinerman RA, et al: Risk of cutaneous carcinoma in patients treated with oral methoxsalen photochemotherapy for psoriasis. N Engl J Med 300:809–813, 1979

(*4*) Melski J, Tanenbaum L, Parrish JA, et al: Oral methoxsalen photochemotherapy for the treatment of psoriasis: A cooperative clinical trial. J Invest Dermatol 68: 328–335, 1977

(*5*) Roenigk HH Jr, Caro WA: Skin cancer in PUVA—48 cooperative study. J Am Acad Dermatol 4:319–324, 1981

(*6*) Sidney B, Herszenson S, Winkelman K: The involuting lichenoid plaque. Arch Dermatol 118:93–98, 1982

(*7*) Goett DK: Benign lichenoid keratosis. Arch Dermatol 116:780–782, 1980

(*8*) Lumpkin LR, Helwig EB: Solitary lichen planus. Arch Dermatol 93:54–55, 1966

(*9*) Weedon D, Searle J, Kerr JF: Apoptosis: Its nature and implications in dermatopathology. Am J Dermatopathol 1:133–404, 1979

(*10*) Kumakiri M, Hashimoto K: Histogenesis of primary localized cutaneous amyloid via filamentous degeneration. J Invest Dermatol 84:1032–1035, 1981

(*11*) Hashimoto K, Kumakiri M: Colloid-amyloid bodies in PUVA-treated human psoriatic patients. J Invest Dermatol 72:70–80, 1979

(*12*) Greene I, Cox AJ: Amyloid deposition after psoriasis therapy with psoralen and long-wave ultraviolet light. Arch Dermatol 115:1200–1202, 1979

(*13*) Harrist TJ, Gonzalez E: Histologic alterations of psoralen- and longwave ultraviolet radiation-exposed skin. Natl Cancer Monogr 66:197–204, 1984

(*14*) Gschnait F, Wolff K, Hönigsmann H, et al: Long-term photochemotherapy: Histopathological and immunofluorescence observations in 243 patients. Br J Dermatol 103:11–22, 1980

Histologic Alterations of Psoralen- and Longwave Ultraviolet Radiation-exposed Skin[1]

Terence J. Harrist[2] and Ernesto Gonzalez[3]

ABSTRACT—Histologic changes in skin exposed to psoralen photochemotherapy (PUVA) may be classified as acute or chronic. The acute set of histologic alterations includes changes associated with lesion regression and PUVA toxicity. The chronic set of changes related primarily to the side effects of PUVA therapy. In psoriatic epidermis, morphologic evidence of cellular hyperactivity ceases with accompanying loss of psoriasiform hyperplasia, whereas in other inflammatory disorders the lymphocytic infiltrate is cleared in the epidermis and papillary dermis. In short-term therapy, changes include melanocytic hyperplasia and increased activity with marked keratinocyte hypermelaninosis. In skin chronically exposed to PUVA, all cutaneous compartments are affected with an epidermal maturation disorder, melanocyte activation, as well as dermal collagen and elastic tissue degeneration, some of which persist after cessation of therapy. — Natl Cancer Inst Monogr 66: 197-204, 1984.

A variety of histologic alterations, observed in PUVA-exposed skin (*1*), may be separated into the histologic changes associated with lesion regression and those associated with PUVA therapy itself. With respect to the first point, Braun-Falco et al. (*2*) found that psoriatic plaques treated with PUVA underwent an orderly sequence of histologic changes during regression, e.g., the stratum granulosum forms with subsequent restoration of the stratum corneum. The psoriasiform hyperplasia is lost as the inflammatory infiltrate subsides, but the resulting skin appeared essentially normal in this study of short-term PUVA therapy. These light microscopic findings were confirmed by 2 electron microscopic studies. In the first, Tsuji and Cox (*3*) found that after clearing of the lesions under PUVA therapy, keratinocytic nuclei were similar to normal except for a relative increase in heterochromatin. The enlarged nucleoli, nuclear bodies, and nucleolar fibrillary centers of psoriatic nuclei, which are ultrastructural evidence of cellular hyperactivity, had resolved. Hashimoto and his colleagues (*4*) made similar observations and also noted the loss of widened intercellular spaces and formation of cytoplasmic lipid vacuoles.

When the lesions of lichen planus and mycosis fungoides are treated with PUVA (*5, 6*), loss of the superficial bandlike infiltrate occurs, probably due to both inhibition of mitosis and cytolysis. There is usually superb clearing of epidermal lymphoid cells and markedly decreased numbers of papillary dermal lymphoid cells; however, deeper infiltrates are much more refractory. In vitiligo, stimulation of the follicular melanocyte pool with hyperplasia and centrifugal spread into periacrotrichial epidermis appear to result in repigmentation (*7*). Thus in various disorders beneficial clinical effects due to PUVA result, and their mechanisms have been studied histologically and ultrastructurally.

Histologic alterations develop in short-term and chronically PUVA-treated skin that correlate with such side effects as the bronze tan; morphologically, they may form a basis for other less acceptable sequelae. In PUVA-exposed skin, all the features listed below have been noted in the epidermis in varying frequency. Hyperorthokeratosis, with preserved basket-weave pattern uniformly present (fig. 1), tends to diminish in patients with low frequency therapy (*1, 8, 9*). Parakeratosis usually due to exacerbating psoriasisis is only rarely identified. Apoptosis has also been noted with production of so-called dyskeratotic or sunburn cells (fig. 2). An increased number (0.25–1/mm) of apoptotic cells was noted in all biopsies of PUVA-treated nonlesional skin when compared with non-PUVA-treated normal skin (0.1/mm) of 20 psoriatics (unpublished observations). The number of apoptotic cells decreased with length of therapy and total PUVA dosage. Observed infrequently is a slightly disordered epidermal maturation, a minimal alteration in the orderly sequence of keratinocyte maturation as is frequently seen in a large number of inflammatory disorders, such as erythema multiforme, lupus erythematosus, and graft versus host disease (*8*). This maturation disorder is not the irregular atypical maturation noted in actinic

Epidermis	Dermis
Hyperkeratosis	Subepidermal hyalinization
Apoptosis	Dermal melanophages
Epidermal maturation disorder	Colloid–amyloid bodies
	Elastic tissue degeneration
Increased keratinocytic melaninization	Endothelial activation
	Perivascular fibrillary substance
Melanocytic activation	
Melanocytic hyperplasia	Reticular dermal collagen alterations
Keratinocyte "atypia"	

ABBREVIATIONS: PUVA = psoralen and UV radiation at 320–400 nm; J = joule(s); H & E = hematoxylin and eosin.

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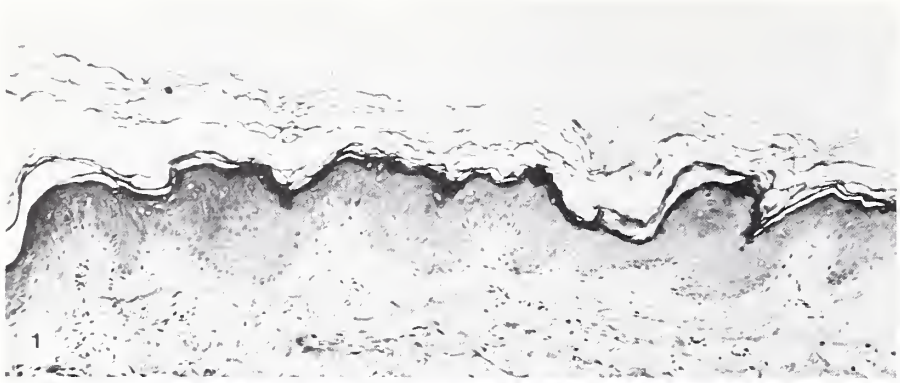

FIGURE 1.—Basket-weave hyperorthokeratosis is approximately as thick as the underlying malpighian layer. H & E. × 100

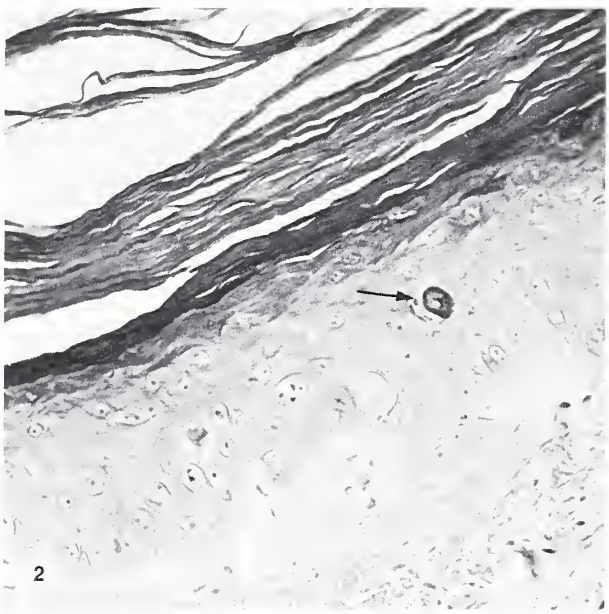

FIGURE 2.—An apoptotic keratinocyte (*arrow*) resides in an epidermis with slight maturation disorder. *Note* the enlarged nuclei with multiple nucleoli and irregularity of cellular size in the stratum spinosum. H & E. × 400

FIGURE 3.—Numerous dermal melanin-laden macrophages (*arrowheads*) are present in the papillary and upper reticular dermis. Markedly increased epidermal melanin extends into the stratum corneum (*arrow*) H & E. × 200

keratoses. Gschnait and his colleagues (*1*) did not report this observation in their study of 243 long-term PUVA-treated patients.

Uniformly observed in PUVA-treated psoriatic patients is increased keratinocytic melaninization (*1, 9*). Increased quantities of melanin (fig. 3) are present not only in the basal unit but are also noted in the upper epidermal layers, even the stratum corneum (*1, 8*).

Increased epidermal melanin implies increased melanocytic activity, which has been confirmed in several studies. Increased 3,4-dihydroxyphenylalanine reactivity of each melanocyte (*10, 11*), increased numbers of melanosomes, and increased transfer of melanosomes to keratinocytes have been documented ultrastructurally. It is debatable whether an increase in individual melanosome size occurs (*12–14*).

Melanocytic hyperplasia is frequently (75–100%) found in long-term PUVA-treated patients with psoriasis (fig. 4). No melanocytic degenerative alterations were noted in PUVA-treated psoriatics who had received 40–396 J UVA/cm^2 (*4, 12, 15*). However, in a study of 17 patients with UVA doses ranging from 230 to 3,500 J/cm^2, degenerative changes, including disrupted mitochondria, large

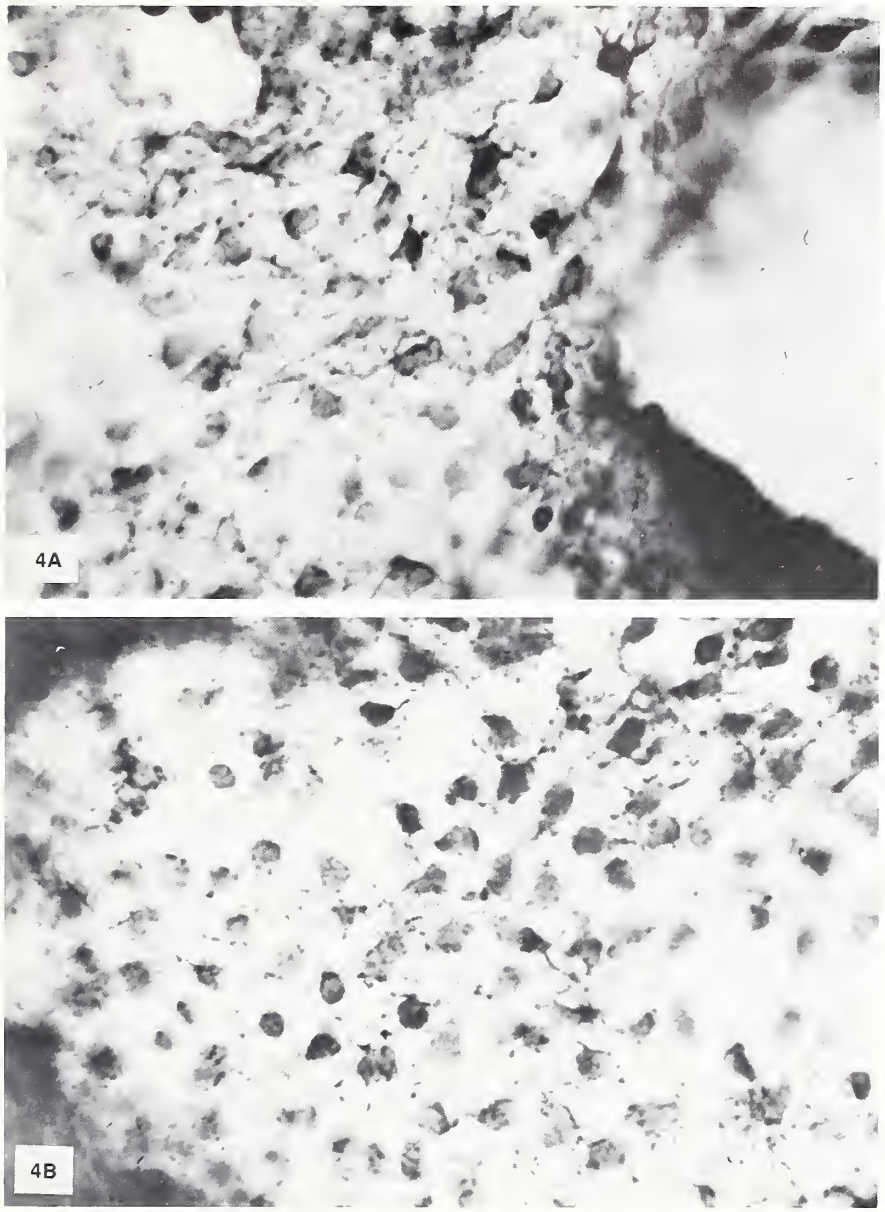

FIGURE 4.—DOPA-incubated epidermal sheets. A) Normal non-PUVA-treated skin. Tyrosinase activity of individual melanocytes is measured by the quantity of black reaction product within each cell. × 400. B) PUVA-treated skin. *Note* the greater density of melanocytes after PUVA therapy and the greater quantity of reaction product (courtesy of Dr. M. Pathak)

lysosomes, and cytoplasmic vacuoles, were noted and persisted in an unspecified number of patients up to 15 months after cessation of therapy (*16*).

Keratinocytic atypia, similar to that in actinic keratoses, may be observed in the clinically normal, relatively non-light-exposed skin of PUVA-treated patients. In the 243 patients studied by Gschnait and colleagues (*1*), only 1 had atypia of keratinocytes noted. However, this patient also had arsenic therapy 15–20 years previously. In another study, 2 of 20 patients had similar foci of epidermal atypia and again 1 of the 2 also had a history of arsenic intake. Braun-Falco et al. (*2*) noted similar "bowenoid" changes in clinically normal skin in 3 of 54 subjects. Of these 3, 2 had a history of arsenic intake. Bergfeld (*9*) noted no such changes in 43 patients studied. Thus of these 360 patients, 6 (<1%) had keratinocytic atypia manifest in clinically normal, non-sun-exposed skin. If those with histories of arsenic intake are excluded, only 2 of 356 (<0.5%) had significant keratinocytic atypism.

In 37 patients studied, Cox and Abel (*17*) report "epidermal dystrophy" in 19 of 37 biopsies from sun-protected skin and 20 of 36 biopsies in sun-exposed skin. The mild epidermal atypia they report corresponds to what we label as slight maturation disorder, which is akin to that observed in many inflammatory dermatoses. Thus significant keratinocytic atypia, in our opinion, was encountered only in those biopsy specimens labeled severe epidermal dystrophy, i.e., 4 of 37 in non-sun-protected skin and 7 of 36 in sun-exposed skin. Importantly, a history of arsenic intake was not reported in this study. Moderate to severe solar elastosis was present in the sun-exposed skin of all 7 subjects in whom severe atypia was noted, possibly implicating sunlight or previous 290- to 320-nm radiation therapy in the production of these changes. These con-

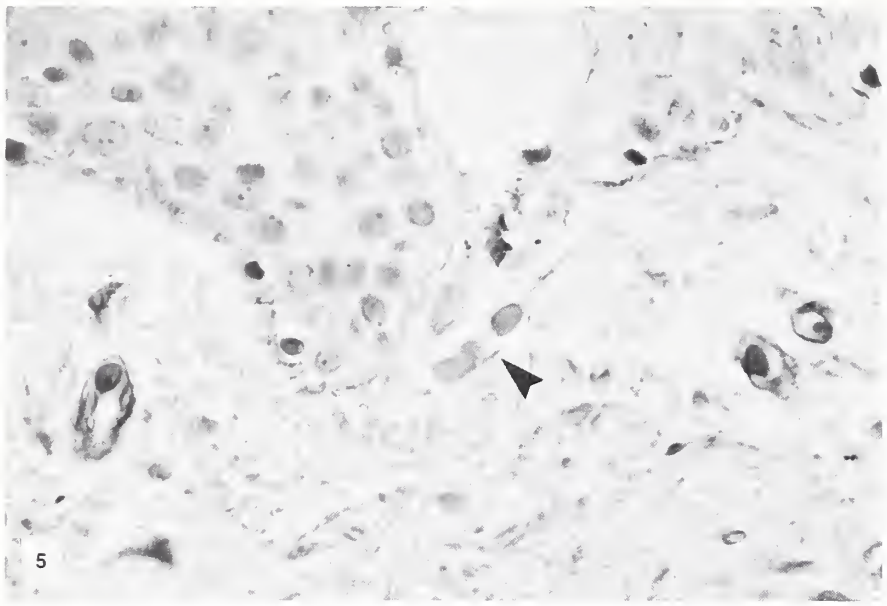

FIGURE 5.—Three colloid-amyloid bodies (*arrowhead*) rest in the dermis near the epidermal basement membrane zone. Some of these bodies exhibit histochemical properties of amyloid. Periodic acid-Schiff. × 400, enlarged four times

siderations are stressed because often the term "epidermal dystrophy" is equated with bowenoid or actinic (premalignant) changes, which we believe is not justified.

Amyloid bodies (fig. 5) have been observed in the superficial dermis of 16% of patients at the end of 1 year of PUVA therapy and 38% at the end of 2 years (*18*). Apoptosis is a process of cellular necrosis in which a neutrophil response is not generated, in contrast to coagulative necrosis (*19*). Apoptosis is the first step in colloid-amyloid body formation (*20*). Necrotic cells, primarily keratinocytes at the dermo-epidermal junction, undergo fibrillary transformation and become incorporated into the dermis, giving rise to 15- to 30-μm diameter opaque periodic acid Schiff-positive and diastase-resistant bodies. These bodies may be metachromatic with crystal violet or exhibit green birefringence after Congo red staining, thus fulfilling generally accepted histochemical criteria for amyloid. Ultrastructurally, these amyloid bodies are composed of straight nonbranching, nonanastomosing fibrils 60–70 angstroms in diameter characteristic of amyloid, regardless of its origin (*20*).

The principal dermal changes associated with PUVA therapy are recorded (*see* p. 197). Increased numbers of dermal macrophages (fig. 3) have been noted in 20% of 243 patients in long-term follow-up (*1*). However, in a study conducted at Massachusetts General Hospital in which biopsies of normal skin were evaluated in comparison with PUVA-treated skin, 16 of 20 patients had increased numbers of melanophages (unpublished observations).

In long-term, PUVA-treated non-repigmented skin of vitiligo patients (*8, 21*), marked subepidermal hyalinization was apparent in virtually all biopsy specimens (fig. 6). Indeed, marked dermal sclerosis was present in the deeper dermis as well (fig. 7). In PUVA-treated psoriatic patients, subepidermal hyalinization is noted in 15–30% of patients (*1–10*). However, the patients with vitiligo had much greater UVA doses, longer duration of therapy, and had no protective pigment (melanin) in the areas biopsied. Also, the subepidermal homogenization or hyalinization, when observed in psoriatic patients, was much less in degree. Ultrastructurally, this change is due to loss of elastic tissue, proliferation of basal lamina, and deposits of finely fibrillary material as shown in figure 8 (*1*).

Although virtually no elastic fiber abnormalities in the deeper dermis have been identified in psoriatic patients when light microscopy is used, fragmentation and irregularity of elastic fibers were noted frequently in patients with vitiligo.

In ultrastructural studies by Zelickson and co-workers (*22*), elastic fiber alterations in PUVA-treated psoriatics included decreased elastin, discontinuity and loss of parallel array of microfibrils, vesicle formation, and elastic fiber fragmentation. The changes begin superficially and then develop in the deeper elastic fibers, persisting after cessation of therapy.

Under light microscopy, 20 patients showed evidence of variable vessel ectasia, slight vessel wall thickening, and perivascular edema when compared with normal skin. Ultrastructurally, endothelial activation and degeneration with basal lamina duplication has been noted. Hashimoto et al. (*4*) noted perivascular deposition of a fibrillary substance in skin exposed to either UVA or PUVA. Increased transcapillary escape rate of albumin in PUVA-treated patients indicates that at least a portion may be fibrillary plasma proteins. Using direct immunofluorescence techniques, we observed fibrin deposition in superficial perivascular spaces (unpublished observations).

Thus morphologic alterations of many of the components of skin have been documented: the increased melanin present correlating with the bronze tan, the keratinocytic atypia possibly reflecting increased neoplastic potential, and the melanocytic hyperplasia possibly forming the basis for PUVA lentigo formation (*13*). The significance to the patient of the dermal alterations remains an issue that may be resolved as longer follow-up data are obtained.

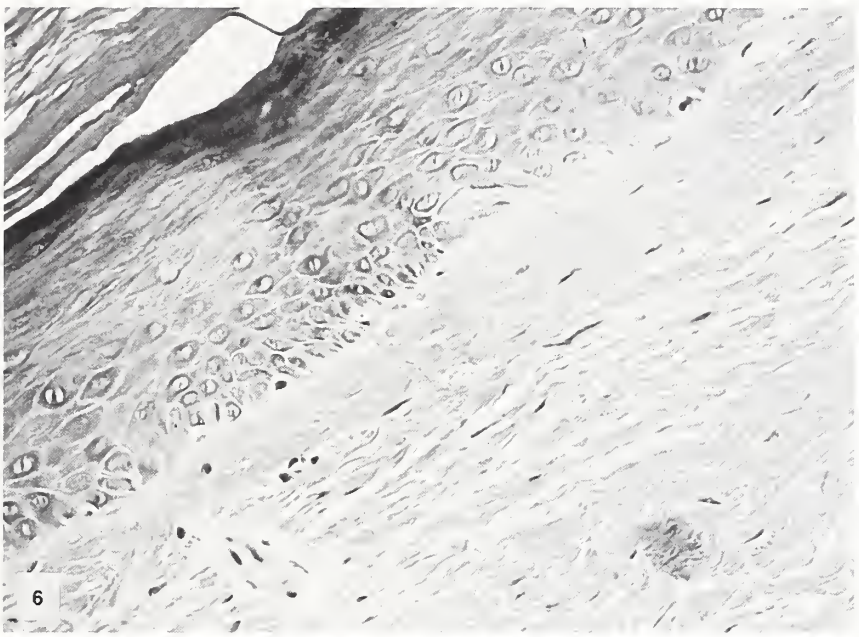

FIGURE 6.—Biopsy of non-repigmented skin of a PUVA-treated patient with vitiligo; marked subepidermal sclerosis is observed. H & E. × 400

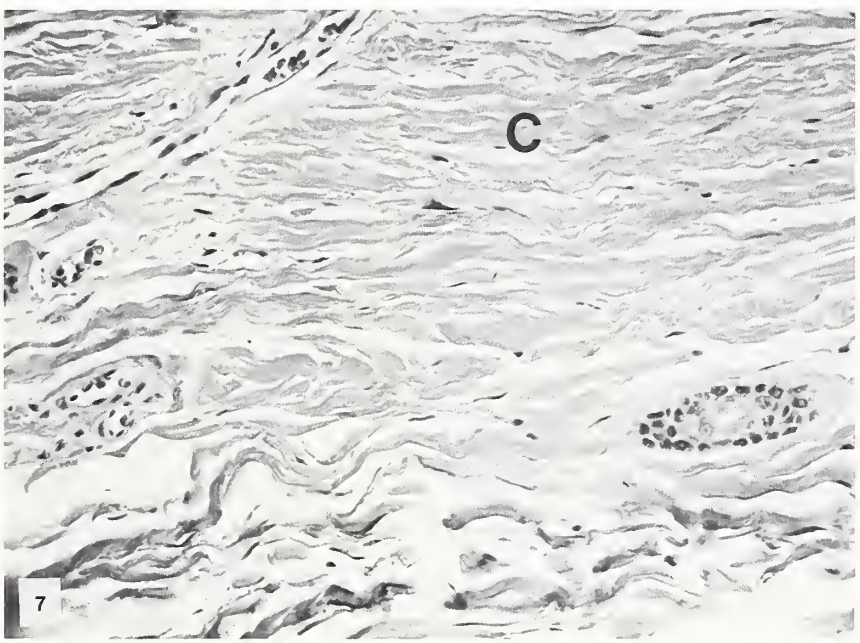

FIGURE 7.—Extensive dermal sclerosis was manifest by abnormal size and arrangement of collagen fibers (C) in vitiligo patients undergoing long-term PUVA therapy. H & E. × 200

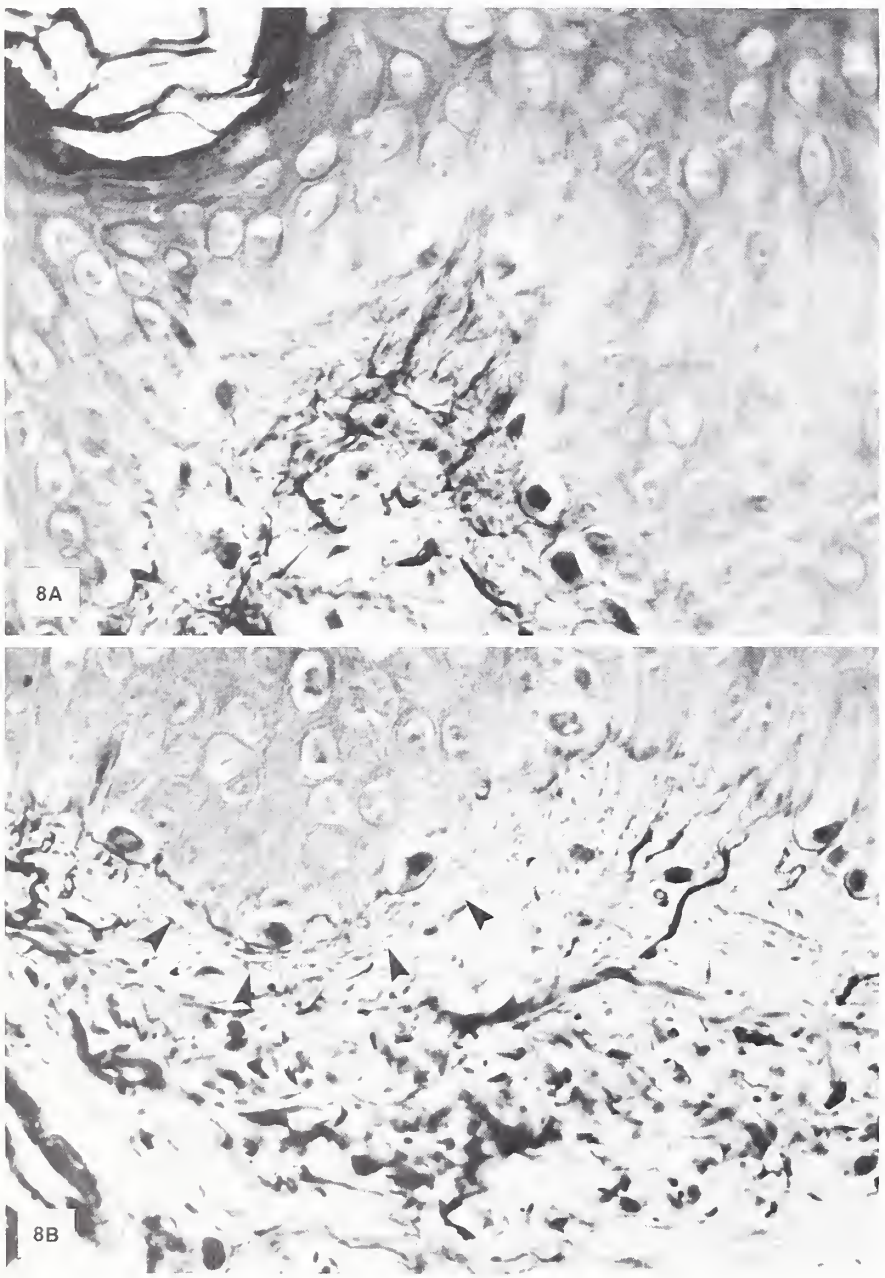

FIGURE 8.—Elastic tissue histochemical preparation. A) Normal skin. Numerous small elastic fibers extend from the dermis to the epidermal basement membrane zone. B) PUVA-treated skin. *Note* absence of elastic fibers along a zone adjacent to the epidermal basement membrane zone (*arrowheads*). Van Gieson × 400, enlarged four times

REFERENCES

(1) Gschnait F, Wolff K, Hönigsmann H, et al: Long-term photochemotherapy: Histopathological and immunofluorescence observations in 243 patients. Br J Dermatol 103:11–22, 1980

(2) Braun-Falco O, Hoffman C, Plewig G: Feinge webliche Veranderungen unter Photochemotherapie der Psoriasis: Eine histologische und histochemische Studie. Arch Dermatol Res 257:307–317, 1977

(3) Tsuji T, Cox AJ: Ultrastructural changes in nuclei and nuceoli in psoriatic epidermis during and after PUVA therapy. Acta Derm Venereol (Stockh) 58:383–389, 1978

(4) Hashimoto K, Kohda H, Kumakiri M, et al: Psoralen–UVA-treated psoriatic lesions. Ultrastructural changes. Arch Dermatol 114:711–722, 1978

(5) Ortonne JP, Thivolet J, Sannwald C: Oral photochemotherapy in the treatment of lichen planus—clinical results, histological and ultrastructural observations. Br J Dermatol 99:77–88, 1978

(6) Lowe NJ, Cripps DJ, Dufton PA, et al: Photochemotherapy for mycosis fungoides—a clinical and histological study. Arch Dermatol 94:50–53, 1959

(7) Ortonne JP, MacDonald DM, Micoud A, et al: PUVA-induced repigmentation of vitiligo: A histochemical (split-DOPA) and ultrastructural study. Br J Dermatol 101:1–12, 1979

(8) Mosher DB, Pathak MA, Harrist TJ, et al: Development of cutaneous lesions in vitiligo during long-term PUVA therapy. Clin Res 28:575, 1980

(9) Bergfeld WF: Histopathologic changes in skin after photochemotherapy. Cutis 20:504–507, 1977

(10) Jimbow K, Kaidbey KH, Pathak MA, et al: Melanin pigmentation stimulated by UV-B, UV-A, and psoralens. J Invest Dermatol 62:548, 1974

(11) Kaidbey KH, Kligman AM: Photopigmentation with trioxsalen. Arch Dermatol 109:674–677, 1974

(12) Rosdahl IK, Swanbeck G: Effects of PUVA on the epidermal melanocyte population in psoriatic patients. Acta Derm Venereol (Stockh) 60:21–26, 1980

(13) Rhodes AR, Harrist TJ, Stern RS, et al: The PUVA lentigo: Increased number of large, dopa-positive melanocytes. J Invest Dermatol 78:357, 1982

(14) Toda K, Pathak MA, Parrish JA, et al: Alteration of racial differences in melanosome distribution in human epidermis after exposure to ultraviolet light. Nature 236:143–146, 1972

(15) Zaynoun S, Konrad K, Gschnait F, et al: The pigmentary response to photochemotherapy. Acta Derm Venereol (Stockh) 57:431–440, 1977

(16) Zelickson AS, Mottaz JH, Muller SA: Melanocyte changes following PUVA therapy. J Am Acad Dermatol 1:422–430, 1979

(17) Cox AJ, Abel EA: Epidermal dystrophy. Occurrence after psoriasis therapy with psoralen and long-wave ultraviolet light. Arch Dermatol 115:567–570, 1979

(18) Greene I, Cox AJ: Amyloid deposition after psoriasis therapy with psoralen and long-wave ultraviolet light. Arch Dermatol 115:1200–1202, 1979

(19) Hashimoto K, Kumakiri M: Colloid-amyloid bodies in PUVA-treated human psoriatic patients. J Invest Dermatol 72:70–80, 1979

(20) Kumakiri M, Hashimoto K: Histogenesis of primary localized cutaneous amyloidosis: Sequential change of epidermal keratinocytes to amyloid via filamentous degeneration. J Invest Dermatol 73:150–162, 1979

(21) Harrist TJ, Pathak MA, Mosher DB, et al: Chronic cutaneous effects of long-term psoralen and ultraviolet radiation therapy in patients with vitiligo. Natl Cancer Inst Monogr 66:191–196, 1984

(22) Zelickson AS, Mottaz JH, Zelickson BD, et al: Elastic tissue changes in skin following PUVA therapy. J Am Acad Dermatol 3:186–192, 1980

DISCUSSION

J. H. Epstein: Are there any questions for Dr. Harrist?

J. E. Hearst: I would like to ask you about the reversibility of these vascular changes after PUVA treatment and also if you would make some comments comparing the changes you saw with those seen in actinic degeneration and chronic sun damage.

T. J. Harrist: In reports concerning the histologic alterations in PUVA-treated skin, I find it difficult to sort out whether the alterations are due to PUVA, other forms of therapy (such as 290- to 320-nm phototherapy), or a summation of all the therapy used. However, in the PUVA patients studied by Gonzalez, Lopansri, Mihm, Parrish, and me, no solar elastosis was identified in sun-protected skin of those patients who had been given PUVA alone. Solar elastosis was apparent universally in those patients who had previous therapy with 290–320 nm UV radiation. This observation is corroborated by the study of Gschnait et al.

Epidermal dystrophy, as I mentioned in my discussion, is not equivalent to an actinic keratosis histologically. In my opinion, the term "epidermal dystrophy" does not imply premalignant change. For example, when Momtaz, Parrish, and I studied PUVA phototoxic reactions, marked epidermal and keratinocytic atypia were observed that resolved over the course of 3 weeks. Thus both the epidermal alterations and dermal changes observed with PUVA appear to be different from actinic damage.

Elastic tissue alterations have been attributed to PUVA. In contrast to solar elastosis, no subepidermal zone of sparing exists. In PUVA, elastic tissue degeneration is most evident in the elastic fibers (oxytalan) immediately subjacent to the epidermal basement membrane zone. The elastic tissue and melanocytic alterations appear to persist after cessation of therapy. To my knowledge, the reversibility of the other changes has not been studied.

K. M. Halprin: I think some of those symptoms of Bowen's disease in patients from Braun-Falco's group disappeared with further therapy.

Harrist: Yes. We have noted that some phototoxic drug eruptions may induce marked epidermal atypia and disarray that resolves. Therefore, a precedent for light-induced atypia to resolve is apparent.

Halprin: So even calling it Bowen's disease may not be correct.

G. Lazarus: Melanophages are called melanophages because they contain melanin. That does not mean melanin is the only thing they contain. The kind of elastin resorption you are seeing is the kind of reaction one actually would expect from an active (phagocytic) process.

Harrist: It is true that macrophages contain elastase. In

fact, one cause of anetoderma is giant cell-mediated elastolysis. However, no macrophage infiltration has been observed in PUVA-treated skin.

Lazarus: Have you, under any circumstances, observed in either acute or chronic toxicity induction of polymorph accumulation within epidermis or within the connective tissue?

Harrist: In the study that Drs. Rosario, Mark, Mihm, and Parrish conducted on acute PUVA toxicity, neutrophils, nuclear debris, and edema were noted about vessels but not in the connective tissue or epidermis. Momtaz, Parrish, and I made the same observation.

Lazarus: Is there an induction of endothelial necrosis in those situations you described?

Harrist: Endothelial necrosis may be observed after PUVA phototherapy.

H. H. Roenigk, Jr: I would like to ask if you have investigated the mucopolysacchrides in the dermis? In the March 1982 issue of *The Journal of the American Academy of Dermatology,* Drs. Levin, Caro, and I reported on increased acid mucopolysacchrides in early PUVA therapy. When we checked later, after PUVA had been stopped, it disappeared completely.

Epstein: Deposition was normal. Was it normal in the papillary dermis?

Roenigk: No, throughout the entire dermis.

Harrist: Dr. Bergfeld noted increased dermal mucin that was detected histochemically, probably presenting hyaluronic acid produced by fibroblasts.

S. Lerman: Dr. Harrist, I am curious about the amyloid bodies you showed. You indicated that they are made up of regularly arranged beta-pleated configurations of some protein component. I assume that amyloidosis is sort of a breakdown product. Is it unusual to get this type of careful beta-pleated configuration in a breakdown product? This has probably been present previously. How did you decide this was a beta-pleated configuration?

Harrist: These bodies have all the histochemical and electron microscopic properties of amyloid. The exact steric configuration of the amyloid deposits in PUVA-treated skin has not been studied. However, all amyloid, whether immunologic or endocrine protein, has had a beta-pleated sheet arrangement, and so, I assume, will the PUVA amyloid bodies.

Lerman: How do they become beta-pleated?

Harrist: The exact mechanisms of amyloid body formation in cutaneous disorders is unknown. Hashimoto initially indicated that the protein was made by fibroblasts. His recent work on ultrastructural primary cutaneous amyloidosis has shown an origin from epidermal fibrillary proteins, probably epidermal keratins. These proteins appear to undergo macrophage processing.

Carcinogenic Effects of Monofunctional and Bifunctional Furocoumarins[1, 2, 3]

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ABSTRACT—We initiated these studies to determine whether bifunctional (interstrand cross-linking) psoralens, such as 8-methoxypsoralen (8-MOP), are more carcinogenic than are the monofunctional, such as angelicin or isopsoralen derivatives, and 3-carbethoxysporalen (3-CP). Hairless mice (Skh:hr-1) in groups of 40 were treated three times weekly for 12 to 15 months. There were 17 groups, and the photocarcinogenic effects of 5 psoralens [8-MOP, 3-CP, 5-methylangelicin, 4,5′-dimethylangelicin (4,5′-DMA), and angelicin] were investigated. Ethanolic solutions of 0.01–0.1% psoralens were topically applied at 5.0 or 50 $\mu g/cm^2$ from cervical to lumbar regions 45 minutes before exposure to UVA (320–400 nm) radiation (0.1, 2.5, or 7.5 joules/cm^2). Control groups received either the drug application or UVA exposure only. The study revealed that isopsoralens, such as 5-methylangelicin or 4,5′-DMA, that form monofunctional adducts are more carcinogenic than bifunctional psoralens. The latency and time required for 50% prevelance of tumors was much longer with 8-MOP than with 4,5′-DMA or 5-methylangelicin. Mice treated with the latter 2 compounds had a greater number and larger tumors than mice treated with 8-MOP. The monofunctional angelicin was weakly carcinogenic, whereas 3-CP, also a monofunctional psoralen, was noncarcinogenic. Histologic examination revealed that tumors induced by 8-MOP, 5-methylangelicin, or 4,5′-DMA were all squamous cell carcinomas. Because of their skin-photosensitizing property and their ability to induce interstrand cross-links and severe damage to DNA in replication, bifunctional psoralens apparently produce more lethal damage in cells than do monofunctional isopsoralens. Hence 8-MOP is less carcinogenic than the monofunctional isopsoralens, which are nonphotosensitizing and presumably evoke more error-prone repair. — Natl Cancer Inst Monogr 66: 205–210, 1984.

Certain linear furocoumarins, such as 8-MOP, 4,5′,8-trimethylpsoralen, etc., are widely used in the treatment of psoriasis, pigmentary disorders (e.g., vitiligo or leukoderma), mycosis fungoides, and other skin diseases. Most of the linear psoralens are potent photosensitizing molecules. Upon irradiation with UVA, their conjugated tricyclic structure enables them to absorb the radiation and thereby generate metastable, highly reactive triplet states which further react with components of a biologic system, producing detectable cellular and biochemical changes (*1*).

Psoralen photoreactions can lead to the formation of psoralen dimers (*1*), covalent protein adducts (*2*), and most importantly, for therapeutic use in diseases of rapid cell proliferation, monofunctional and bifunctional adducts to pyrimidine bases of DNA (*3, 4*). Furocoumarins, such as 8-MOP, form cyclobutane (C-4) covalent monoadducts with pyrimidine bases in cellular DNA upon irradiation; some of these monoadducts do undergo an additional photoreaction leading to the formation of bifunctional adducts (interstrand cross-links) between the 2 opposite strands of DNA. Angular psoralens, i.e., angelicin or isopsoralen, due to their steric configuration, and linear psoralens, due to the presence of a carbethoxy-blocking group, are prevented from forming interstrand cross-links in DNA, and, therefore, are designated as monofunctional psoralens. The mechanism of action in psoralen photochemotherapy of psoriasis is believed to be due to its ability to conjugate with cellular DNA and inhibit DNA, RNA, and protein synthesis. This inhibition is in agreement with psoralen photobinding capacity and not due to the type of monofunctional or bifunctional adducts (*5, 6*).

Although the therapeutic potential of bifunctional psoralens is well recognized, these molecules have been associated with such adverse acute cutaneous effects as phototoxicity (erythema and edema) and inflammatory changes and chronic effects like skin cancer and aging of the skin (*7, 8*). Monofunctional psoralens, however, although displaying a high DNA-binding capacity, are recognized to be noninflammatory and nonerythemogenic (*9*). Recently, Dubertret et al. (*10*) reported that 3-CP, a linear and nonphotosensitizing agent useful in treatment of psoriasis and exhibiting a high photoaffinity to DNA, was noncarcinogenic in mice when tested either by topical application or after ip injection. Bordin and associates (*5, 6, 11*) reported the

ABBREVIATIONS: 8-MOP = 8-methoxypsoralen; UVA = UV radiation at 320–400 nm; 3-CP = 3-carbethoxypsoralen; 4,5′-DMA = 4,5′-dimethylangelicin; PUVA = psoralen plus UVA; T_{50} = time required to produce 50% tumor prevalence in surviving animals; UVB = UV radiation at 290–320 nm.

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[2] Supported in part by Public Health Service grant 5-R01-CA05003-24 from the National Cancer Institute and a grant from the Paul B. Elder Company, Bryan, Ohio.

[3] The 5-methylangelicin and 4,5′-dimethylangelicin used in the experiments contained extremely small amounts of linear isomers formed during their chemical synthesis (U.S. Patent No. 4312883); these isomers can markedly affect the photobiologic activity of the 2 compounds (Rodighiero G, Guiotto A, Pastorini G, et al: Farmaco [Sci] 36:648–661, 1981). In fact, highly purified 4,5′-dimethylangelicin showed no capacity to induce skin cancer in albino hairless mice (Dall'Acqua F, Young R: Unpublished results).

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synthesis of certain nonlinear psoralens or methylated angelicin derivatives, such as 5-methylangelicin and 4,5′-DMA. These alkyl-substituted angelicins exhibited: 1) an increased photoreactivity toward DNA, 2) little or no cutaneous photosensitization reaction, and 3) antiproliferative activity to the extent of good-to-excellent therapeutic effects in clearing psoriatic plaques after 8 to 16 treatment sessions (*9*) with topically applied 5-methylangelicin, 4,5′-DMA, and 4,5-dimethylangelicin.

Until recently, the carcinogenic activity of furocoumarins was believed to be due to their skin-photosensitizing property, ability to react with DNA and to form interstrand cross-links, and ability to induce error-prone repair. Because isopsoralens were nonerythemogenic and therapeutically useful in psoriasis but less damaging to DNA, we decided to examine the carcinogenic effects of monofunctional angelicin or isopsoralen in a hairless mouse model. In the studies presented, the carcinogenic potential of 5-methylangelicin, 4,5′-DMA, angelicin, and 3-CP was evaluated and compared with that of bifunctional 8-MOP.

MATERIALS AND METHODS

Animals.—We obtained Skh:hairless-1 male albino mice from Dr. S. J. Mann, Temple University Health Center (Philadelphia, Pa.), that were 6 weeks old when the studies began. Mice were housed 4 to a cage, fed Purina chow and water ad libitum, and shielded from light for the duration of the experiments (12–15 mo). Those in the experimental groups of 40 were treated with both drug and UVA exposure, whereas the control groups of 20 received either the drug or UVA exposure alone (table 1). We had a total of 17 groups; the study was done in 2 phases at 2-month intervals with appropriate controls.

Light source.—In each study, the UVA source included a battery of 6 FR40T12/PUVA Sylvania Lifeline bulbs obtained from GTE Sylvania–Emissive Products (Exeter, N.H.) emitting radiation between 320 and 400 nm with a maximum wavelength of 355 nm. Animals placed in wired cages (4/cage) were irradiated at a distance of 6 cm from a planar array of 6 PUVA lamps shielded by an 0.005-inch thick Mylar sheet so that wavelengths shorter than 320 nm were eliminated. Lights were metered periodically with a calibrated, cosine-corrected International Light IL700 spectroradiometer (Newburyport, Mass.) equipped with a UVA detecting probe. Exposure doses were initially based on the minimal phototoxic dose of mice receiving topical 8-MOP applications. Subsequently, the exposure dose was incrementally increased to the final dose as indicated in table 1.

TABLE 1.—*Experimental groups in evaluation of the carcinogenic potential of monofunctional and bifunctional psoralens*

Group	Drugs	Concentration, %	Dose of UVA, J/cm^2
A	8-MOP	0.1	2.5
D	〃	0.01	1.0
H	〃	0.1	1.0
I	Angelicin (isopsoralen)	0.1	2.5–5.0
B	4,5′-DMA	0.1	2.5
C	〃	0.1	7.5
E	5-Methylangelicin	0.01	1.0
F	〃	0.1	1.0
G	3-CP	0.1	1.0
J	8-MOP	0.1	None
K	Angelicin (isopsoralen)	0.1	〃
L	4,5′-DMA	0.1	〃
M	5-Methylangelicin	0.1	〃
N	3-CP	0.1	〃
O	None	0.1	1.0
P	None	0.1	2.5
Q	None	0.1	7.5

Drugs.—Crystalline 8-MOP was obtained from Elder Pharmaceuticals (Bryan, Ohio). For ease of topical application, drug concentrations were made up in solutions of absolute ethanol every 14 days and kept shielded from the light. At the start of these studies, the DNA cross-linking potential of these 5 drugs was examined by means of hydroxyapatite chromatography and measurement of spectrophotometric absorption (*12*); DNA cross-linking was determined from 8-MOP. We noted 80–90% of the irradiated DNA (4 J/cm^2) had cross-links; however, no DNA cross-linking was seen for either of the 3 isopsoralens or for 3-CP.

Methods.—Ethanolic solutions of psoralens at varied concentrations from 0.01 to 0.1% were uniformly applied in a volume of approximately 0.05 ml on the backs of the mice below the cervical area to shortly above the tail region and provided a concentration of 5 to 50 μg/cm^2 of skin surface. In specially built wire cages, mice were immobilized and, after a waiting period of 45-minutes post application of the drug (for maximal diffusion of psoralens), they were returned to their housing cages. Treatments were administered three times weekly for the duration of the study (except where noted). Cutaneous phototoxicity reactions and the size and number of tumors for each animal were recorded weekly. Mice were photographed periodically, and biopsies of skin tumors in various growth phases were obtained for histologic examination at regular intervals. Animals were irradiated until the termination of the study.

Tabulation of data.—Tumors were scored as papillomas (benign, possibly premalignant lesions) when they appeared as raised, erythematous, rounded papules, usually less than 3 mm in diameter. They were designated as squamous cell carcinomas when confirmed by histologic analysis and when appearing as markedly differentiated, keratinous, raised, and solid-cored lesions usually ranging from 3.5 mm to 3 cm or more in size.

Histologic procedure.—Biopsy specimens of skin bearing tumors of varying sizes were preserved in 10% buffered formalin, sectioned, and processed for routine hematoxylin and eosin staining. Approximately 50% of the mice from each group were biopsied and examined for histologic changes.

RESULTS

The results of our experiments in which we compared the carcinogenicity of 8-MOP, a bifunctional furocoumarin, with the monofunctional psoralens 4,5′-DMA, 5-methylangelicin, angelicin, and 3-CP are shown in table 2. This table provides the total tumor yield and T_{50} for each of the groups. Figure 1 illustrates a plot of tumor development for the duration of the study. To maintain clarity and appropriate comparison, we plotted selected doses of 8-MOP, 4,5′-DMA, and 5-methylangelicin.

TABLE 2.—*Results of carcinogenicity studies of monofunctional and bifunctional psoralens*

Group	Drugs[a]	UVA dose, J/cm²		Phototoxicity[b]	T_{50}[c]		Tumor yield, %
		Per treatment	Total		Wk	J/cm²	
A	8-MOP	2.5	248.25	++++	60	248.25	54
D	"	1.0	141.5	+++	56	125.5	65
H	"	1.0	220.0	+++	60	180.0	70
I	Angelicin	2.5–5.0	250.0	−	>60	>250	20
B	4,5′-DMA	2.5	289.0	−	40	222.75	97.3
C	"	7.5	758.0	−	42	608.75	100.0
E	5-Methylangelicin	1.0	136.5	−	53	118.5	78
F	"	1.0	107.0	−	33	66.0	97
G	3-CP	1.0	120.0	−	—	—	0.0
J	8-MOP	0.0	0.0	−	—	—	—
K	Angelicin	"	"	−	—	—	—
L	5-Methylangelicin	"	"	−	—	—	—
M	"	"	"	−	—	—	—
N	3-CP	"	"	−	—	—	—
O	None	1.0	148.0	−	—	—	—
P	"	2.5	250.0	−	—	—	—
Q	"	7.5	751.5	±	—	—	—

[a] Groups D and E received 0.01% of the respective drug; all others were given 0.1% except group O–Q which received none.

[b] Symbols: ++++ = marked phototoxicity (deep red erythema, edema, and vesiculation); +++ = strong phototoxicity (erythema and edema); ± = barely perceptible redness; − = no photoxicity.

[c] Dash indicates absence of tumors.

The data in table 2 and figure 1 indicate that 4,5′-DMA and 5-methylangelicin are unquestionably carcinogenic agents, more so than is the bifunctional 8-MOP. This was evidenced by the following supporting observations:

1) At the conclusion of the studies, percent tumor yields in the animal groups given 4,5′-DMA and 5-methylangelicin were higher than in the 8-MOP group.

2) The T_{50} for the 4,5′-DMA and 5-methylangelicin groups was consistently less than that for the 3 groups treated with topical 8-MOP.

3) Mice treated with the 2 angelicin derivatives had a larger tumor size (>1.5 cm) and a greater number of tumors/mouse than mice treated with 8-MOP.

4) Mice treated with 0.01% 5-methylangelicin and low-dose UVA attained T_{50} approximately 20 weeks after mice treated with 0.1% of the drug; however, with 8-MOP the same concentration dependence was not observed.

Interestingly, after 50 weeks of thrice weekly treatment, 3-CP was observed to be a noncarcinogenic psoralen derivative; however, angelicin was a weak carcinogenic agent.

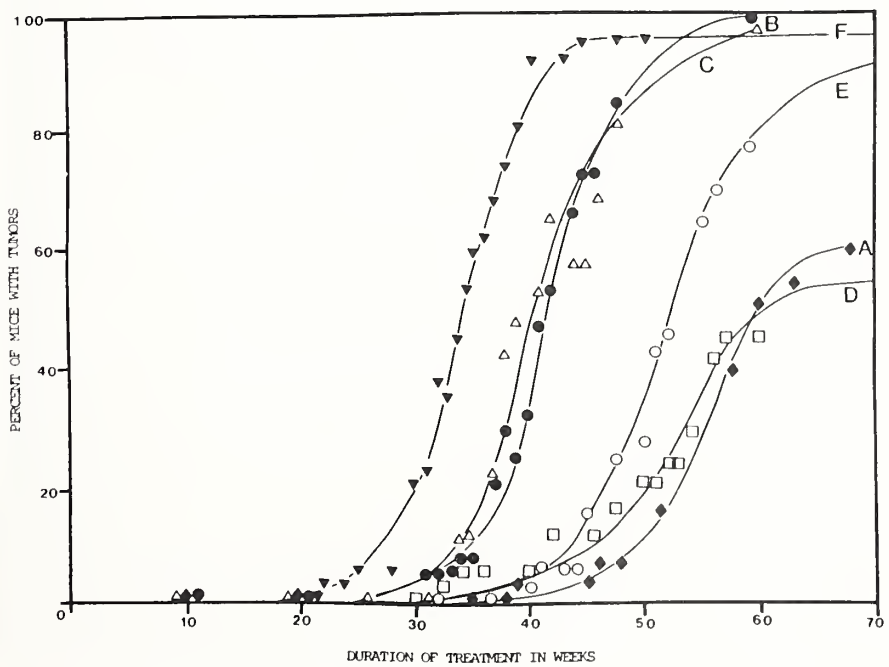

FIGURE 1.—Carcinogenesis of monofunctional isopsoralens (angelicins) and bifunctional 8-MOP. Albino hairless mice were treated topically with either monofunctional or bifunctional psoralens and irradiated with UVA thrice weekly. Figure is a plot of percent tumor yield vs. duration of treatment in weeks. Percent drug and UVA (J/cm²) per group: A) 0.1% 8-MOP + 2.5; B) 0.1% 4,5′-DMA + 2.5; C) 0.1% 4,5′-DMA + 7.5; D) 0.01% 8-MOP + 1.0; E) 0.01% 5-methylangelicin + 1.0; and F) 0.1% 5-methylangelicin + 1.0.

These observations on 3-CP are in complete agreement with those reported by Dubertret et al. (*10*) and by our studies that indicated 3-CP is a highly photolabile compound, and the resulting photoproduct of 3-CP is a poor DNA binding agent (Pathak MA, Joshi PC: Unpublished observations). Animals treated with UVA alone developed no tumors.

Macroscopic Observations

Throughout the course of the studies, 8-MOP- and UVA-treated animals demonstrated marked photosensitivity, which was manifested by erythema, severe pruritus, edema, and heavy scabbing. No photosensitivity (edema or erythema) was observed in groups treated with 4,5′-DMA, 5-methylangelicin, angelicin, and 3-CP or in groups treated with drug or UVA radiation alone. However, all groups receiving UVA light exhibited moderate desquamation, hyperplasia, and thickening of epidermis. Minimal death was noted in certain mice that received UVA treatment and, at the end of 12 to 16 months treatment, survival ranged from 80 to 100% in most of the experimental groups.

Histologic Analysis

Histologic analysis of tumors produced by 8-MOP, 4,5′-DMA, and 5-methylangelicin revealed well-differentiated invasive squamous cell carcinomas displaying a keratocanthoma-like pattern. Several markedly dedifferentiated spindle cell squamous carcinomas were also noted at later stages of the studies. Both 8-MOP- and angelicin-treated epidermis surrounding the tumors displayed atypical epithelial hyperplasia, hyperkeratosis, hypergranulosis, plate-like acanthosis, and frequent areas of necrosis. We explored the possibility of metastasis from these tumors by biopsying most tumor-bearing animals for lymph node, lung, and liver involvement at the time of necropsy, but none was observed. All animals that were exposed to UVA developed lens opacity, which indicated cataractogenesis.

DISCUSSION

That the combined treatment with photoreactive 8-MOP and UVA is mutagenic in bacteria (*13, 14*), yeast (*15*), and cultured human skin fibroblasts (*16*) is well recognized. The 8-MOP and UVA treatment of mammalian cells can cause chromosomal damage (*17*), the induction of gene mutations (*16, 18*), and the production of the malignant transformed phenotype (*19*). From these studies, it would appear that bifunctional 8-MOP plus UVA would be carcinogenic. Indeed, the carcinogenic effect of 8-MOP and other linear psoralens (psoralen and 4,5′,8-trimethylpsoralen) has been documented in albino mice (*4*), and orally administered 8-MOP has been implicated as a carcinogenic agent in humans receiving photochemotherapy for psoriasis (*8*). Inasmuch as the carcinogenic potential of bifunctional psoralens has been well established, assumption or acceptance of the notion that monofunctional psoralens would be noncarcinogenic despite their known photobiologic properties was difficult (*10*); these properties include nonphotosensitization, low reactivity with DNA, and the ability to form only monofunctional adducts with DNA which could be easily excised and repaired by the cell. Hanawalt and his associates (*20, 21*) believe that the interstrand cross-link of 8-MOP with pyrimidine bases in DNA, by its nature, must be a less repairable lesion than is the monoadduct (*20, 21*). Furthermore, persistence of cross-links most likely causes cell lethality, whereas the formation of monoadducts that are not lethal to the cell and lead to error-prone repair appear to cause carcinogenicity. This concept is supported by additional observations reported by several other investigators.

Bridges and co-workers (*22*) conducted a comparative study on the effects of monoadducts and cross-links upon induction of mutation and lethality in various bacterial strains. In an excision-repair deficient strain, these investigators found that the conversion of monoadducts to biadducts or cross-links resulted in a reduction of mutation frequency and a significant increment in lethality. These observations were in agreement with the studies reported by Seki and his associates (*23*). Their data also indicated that the monoadducts of 8-MOP were responsible for mutagenesis, whereas the cross-links of 8-MOP with pyrimidine bases in DNA were contributing to lethality. Pohl and Christophers (*24*) also reported their observations on photoinactivation and recovery in cultured skin fibroblasts from young male guinea pigs after the formation of monofunctional and bifunctional adducts with UVA irradiation in the presence of 8-MOP or angelicin. Compared with 8-MOP, a concentration of angelicin 30 times higher than that of 8-MOP was needed to achieve comparative inhibition rates of DNA synthesis. Complete cellular recovery could be seen when the fibroblasts were treated with angelicin plus UVA or 8-MOP plus 395-nm radiation, a condition favorable for the formation of monoadducts only. In contrast to this, the irradiation schedule with 8-MOP plus UVA that promoted the formation of cross-links contributed increased inhibitory effects on DNA synthesis lasting for more than 4 days. This split-dose treatment contributed to the conversion of some monoadducts to cross-links, a finding that supports the view that cross-links are more effective than monoadducts in killing cells and as inhibitory blocks to replication. We also believe that psoralen–pyrimidine interstrand cross-links are less repairable lesions and more lethal to cells than are monofunctional adducts of psoralens and pyrimidines. The monoadducts of psoralen with pyrimidine bases in DNA should be viewed as pyrimidine dimers induced by UVB irradiation and are subject to repair by an error-prone pathway. We know that UVB is carcinogenic to humans and mice, and the only well-recognized damage in DNA by UVB is the formation of pyrimidine dimers leading to error-prone repair and skin cancer.

Our results clearly illustrate the carcinogenic potential of the monofunctional psoralens angelicin, 5-methylangelicin, and 4,5′-DMA. These observations contradict those recently reported by Dubertret and co-workers (*10*) that monoadduct-forming psoralens are nontumorigenic. Our data indicate that 4,5′-DMA and 5-methylangelicin are as carcinogenic or even more so than 8-MOP and produce 50% more tumors on average 20 weeks earlier than does 8-MOP at the same drug concentration and light dose. Tumors induced by monofunctional psoralens were of the same type (squamous cell carcinoma) and similar histologic characterization as those produced by 8-MOP or by UVB

irradiation. However, at 50 weeks, well past T_{50} for the other monofunctional psoralens, 3-CP had not demonstrated any tumorigenic characteristics.

All monofunctional psoralens tested were nonphotosensitizing and did not produce erythema or edema at the UV doses we used. These results present an interesting contrast to the phototoxicity seen in 8-MOP- plus UVA-treated mice. At concentrations from 0.01 to 0.1%, 8-MOP was highly phototoxic and produced severe erythema, edema, desquamation, erosion, and scabbing. The carcinogenic potential of these psoralen drugs cannot be directly correlated to their erythemogenic properties. These findings lend support to the hypothesis that both DNA photoadducts formed by monofunctional psoralens and bifunctional psoralen (cross-links) undergo an error-prone, excision-repair process which is potentially carcinogenic. The difference in the rate of tumorigenesis induced by monofunctional and bifunctional psoralens may be due to the increased cell lethality associated with bifunctional cross-linking of cellular DNA. It is likely that repeated treatments frequently will result in severe damage to DNA (an event from which the cell cannot easily recover) and thus lead to the inhibition of DNA synthesis, decreased cell replication, increased cell death, and ultimately a decreased incidence of mutation and carcinogenesis. This hypothesis correlates well with the increased necrosis, irritation, desquamation, and scabbing observed in 8-MOP-treated mice.

The rate of tumorigenesis was observed to be independent of light dose per treatment for 4,5′-DMA and directly dependent on drug concentration for 5-methylangelicin. However, the rate of tumor production in mice given 8-MOP appeared to be independent of drug dose per treatment; groups given the 0.01% concentration reached T_{50} 4 weeks earlier than those treated with 0.1%. Both groups receiving 0.01 and 0.1% 8-MOP exhibited severe irritation, scabbing, and epidermal necrosis, which gives additional support to the hypothesis that the increased lethality associated with the formation of bifunctional cross-links may actually reduce the occurrence of error-prone repair and thus limit the rate of carcinogenesis in 8-MOP-treated animals.

Of note is the observation that 3-CP, unlike 4,5′-DMA and 5-methylangelicin, is apparently noncarcinogenic after 50 weeks of application and UV exposure, well past the T_{50} observed for the other monofunctional psoralens. In a recent study (Joshi PC, Pathak MA: Unpublished observations), 3-CP has been observed to be a photolabile compound which, upon irradiation, undergoes rapid transformation to a less reactive molecule. Because of its photolability, it appears that this monofunctional furocoumarin behaves distinctly differently than the other monofunctional psoralens, e.g., 5-methylangelicin and 4,5′-DMA, and is noncarcinogenic. In these studies UVA appeared to be noncarcinogenic.

REFERENCES

(*1*) Scott BR, Pathak MA, Mohn GR: Molecular and genetic basis of furocoumarin reactions. Mutat Res 39:29–74, 1976

(*2*) Yoshikawa K, Mori N, Sakakibara S, et al: Photoconjugation of 8-methoxypsoralen with proteins. Photochem Photobiol 29:1127–1133, 1979

(*3*) Musajo L, Rodighiero G, Caporale G, et al: Photoreactions between skin-photosensitizing furocoumarins and nucleic acids. *In* Sunlight and Man: Normal and Abnormal Photobiologic Responses (Fitzpatrick TB, Pathak MA, Harber LC, et al, eds). Tokyo: Univ Tokyo Press, 1974, pp 369–387

(*4*) Pathak MA, Krämer DM, Fitzpatrick TB: Photobiology and photochemistry of furocoumarins (psoralens). *In* Sunlight and Man: Normal and Abnormal Photobiologic Responses (Fitzpatrick TB, Pathak MA, Harber LC, et al, eds). Tokyo: Univ Tokyo Press, 1974, pp 335–368

(*5*) Bordin F, Carlassare F, Baccichetti F, et al: 4,5′-Dimethylangelicin: A new DNA-photobinding monofunctional agent. Photochem Photobiol 29:1063–1070, 1979

(*6*) Bordin F, Carlassare F, Baccichetti F, et al: 5-Methylangelicin: A new highly photosensitizing angular furocoumarin. Experientia 35:1567–1568, 1979

(*7*) Wolff K, Fitzpatrick TB, Parrish JA, et al: Photochemotherapy of psoriasis with orally administered methoxsalen. Arch Dermatol 112:943–950, 1976

(*8*) Stern RS, Thibodeau LA, Kleinerman RA, et al: Risk of cutaneous carcinoma in patients treated with oral methoxsalen photochemotherapy for psoriasis. N Engl J Med 300:809–813, 1979

(*9*) Pathak MA, Joshi PC: The nature and molecular basis of cutaneous photosensitivity reactions to psoralens and coal tar. J Invest Dermatol 80:66–74, 1983

(*10*) Dubertret L, Averbeck D, Zajdela F, et al: Photochemotherapy (PUVA) of psoriasis using 3-carbethoxypsoralen, a non-carcinogenic compound in mice. Br J Dermatol 101:379–389, 1979

(*11*) Bordin F, Baccichetti F, Carlassare F, et al: Pre-clinical evaluation of new antiproliferative agents for the photochemotherapy of psoriasis: Angelicin derivatives. Farmaco [Sci] 36:507–518, 1981

(*12*) Cech T, Pathak MA, Biswas RK: An electron microscopic study of the photochemical cross-linking of DNA in guinea pig epidermis by psoralen derivatives. Biochim Biophys Acta 562:342–360, 1979

(*13*) Igali S, Bridges BA, Ashwood-Smith MJ, et al: Mutagenesis in *E. coli* photosensitization to near ultraviolet light by 8-methoxypsoralen. Mutat Res 9:289–295, 1970

(*14*) Ashwood-Smith MJ, Poluton GA, Barker M, et al: 5-Methoxypsoralen, an ingredient in several sun-tan preparations, has lethal, mutagenic, and clastogenic properties. Nature 285:407–409, 1980

(*15*) Averbeck D, Chandra P, Biswas RF: Structural specificity in the lethal and mutagenic activity of furocoumarins in yeast cells. Radiat Environ Biophys 12:241–252, 1975

(*16*) Burger PM, Simons JW: Mutagenicity of 8-methoxypsoralen and long-wavc ultraviolet irradiation in diploid human skin fibroblasts. Mutat Res 63:371–381, 1979

(*17*) Ashwood-Smith MJ, Grant EL, Heddle J, et al: Chromosome damage in Chinese hamster cells sensitized to near-ultraviolet light by psoralen and angelicin. Mutat Res 43:377–385, 1977

(*18*) Arlett CF, Heddle JA, Broughton BC, et al: Cell killing and mutagenesis by 8-methoxypsoralen in mammalian (rodent) cells. Clin Exp Dermatol 5:147–158, 1980

(*19*) Evans DL, Morrow J: 8-Methoxypsoralen-induced alterations of mammalian cells. J Invest Dermatol 72:35–41, 1971

(*20*) Hanawalt PC, Su-Chin L, Parson CS: DNA repair responses in human skin cells. J Invest Dermatol 77:86–90, 1981

(*21*) Kaye J, Smith CA, Hanawalt PC: DNA repair in human

cells containing photoadducts of 8-methoxypsoralen or angelicin. Cancer Res 40:692–702, 1980

(*22*) BRIDGES BA, MOTTERSHEAD RP, KNOWLES A: Mutation induction and killing of *Escherichia coli* by DNA adducts and cross-links. A photobiological study with 8-methoxypsoralen. Chem Biol Interact 27:221–233, 1979

(*23*) SEKI T, NOZU K, KONDO S: Differential causes of mutation and killing in *Escherichia coli* after psoralen plus light treatment: Monoadducts and cross-links. Photochem Photobiol 27: 19–24, 1978

(*24*) POHL J, CHRISTOPHERS E: Photoinactivation of skin fibroblasts by fractionated treatment with 8-methoxypsoralen and UVA. J Invest Dermatol 73:176–179, 1979

DISCUSSION

K. Wolff: Dr. Pathak, what is the role of higher phototoxicity in skin inflammation and scarring in relation to tumorigenesis? Would you speculate on that? In Langner's study, the animals did not develop tumors, but they had gross changes in the connective tissue, i.e., scarring and ulceration.

M. A. Pathak: I think we also have the same types of changes. High phototoxicity does not lead to high incidence of carcinogenicity. In my opinion, one can evoke carcinogenicity in the absence of visible cutaneous phototoxicity. However, severe phototoxicity will undoubtedly reduce the total tumor yield. To me, the event of tumor formation is through the mutated cell that survives. Here, even with those mutated cells, we are probably killing more cells every day with a daily treatment of 8-MOP plus UVA. Please note that these animals were irradiated three times a week for at least 48 to 52 weeks.

Wolff: I would like to know whether you are inducing too much cell damage, i.e., too much cell killing by producing too much phototoxicity. Do you have more tumor formation at higher doses?

Pathak: Yes, I find I can change the slope of the 8-MOP curves to have higher percentages of tumor formation if we reduce the UVA dose. Induction times can also be a good guide for the tumorigenic potential of the compound. Severe phototoxicity implies severe damage to the cells and lethality, and therefore less carcinogenicity. So you are asking whether bifunctional psoralens are as carcinogenic as the monofunctional ones. I do not know; I am sure that these monofunctional compounds are not safe. That is all I want to say now. We have a problem. Some investigators have been telling us that monofunctional psoralens, being less carcinogenic, are safe; 3-CP is undoubtedly noncarcinogenic. Therefore, we should recognize their observations as well.

H. C. Wulf: Someone asked about the carcinogenicity of UVA. We made a study of that and found no tumors after UVA was used alone on hairless mice for 9 months. When the mice were irradiated at 3 months with sunlight, about 20% of them developed cancer within 1 year. If the mice after such pretreatment with sunlight received UVA for 2 months, this 20% was raised to 40% after 1 year. When we administered UVA for 4 months after the pretreatment, 80% of the animals had tumors. However, if we filtered the UVA so that it did not contain any UVB, the number of tumors was a little lower, but the carcinogenicity would still be accelerated.

K. Halprin: What would the tumor incidence be after the mice were irradiated with UVB alone during those same periods, or if you had continued it for 3 more months?

Wulf: I do not know.

Halprin: Would it be 100%?

Wulf: Probably.

Halprin: So carcinogenesis would progress more slowly and to a lesser degree with UVA than if you continued to give those mice UVB. Could this be a lag effect of the UVB you gave during the first month that is still continuing and not an additive effect of the UVA?

C. Jansen: I have one general comment that I think is important from the clinical point of view. Dr. Pathak and others have been addressing the possible differences in carcinogenic potential of oral and topical PUVA treatment. Unfortunately, the designation "topical treatment" is imprecise. In animal experiments, it usually refers to painting the skin with concentrated alcoholic solutions of the psoralen, usually in concentrations of 0.1 to 1%. However, in the topical bath–PUVA treatment procedure which is in clinical use in Scandinavia, where I come from, much more dilute solutions of the psoralen are used. In that method, 50 mg 4,5′, 8-trimethylpsoralen or 8-MOP is dissolved in a small amount of ethanol and then diluted in 150 liters of water. The final psoralen concentration is thus 0.3 parts/million, i.e., 0.00003%! If we are going to learn anything about the possible carcinogenic effects of this Scandinavian "topical treatment," the experimentalists in this room should include 0.3-parts/million water solutions of psoralens in their animal carcinogenesis experiments.

Pathak: Thank you for your comments. The Food and Drug Administration protocol requires that 0.1–1.0% solution be used in studies on the carcinogenicity of topical agents.

Carcinogenic Risk of Psoralen Plus Ultraviolet Radiation Therapy: Evidence in Humans[1, 2]

Robert S. Stern[3, 4]

ABSTRACT—Despite differences in study populations, methodologies, and levels of psoralen plus UV irradiation at 320–400 nm (PUVA), results from 4 prospective studies of patients treated with PUVA support the hypothesis that the risk of squamous cell carcinoma is increased with prolonged exposure. Tumors that develop after PUVA exposure behaved like skin tumors associated with sun exposure. In view of the latency for cancer in humans, continued prospective follow-up is required if we are to determine the ultimate carcinogenic risk of this type of therapy. — Natl Cancer Inst Monogr 66: 211–216, 1984.

In 1974, Parrish and co-workers (*1*) demonstrated that oral methoxsalen photochemotherapy was effective in clearing psoriasis. Soon afterward, it became evident that continued treatment was needed to maintain a clinical remission (*2*) and, therefore, patients using PUVA for chronic psoriasis may receive large numbers of treatments over a number of years.

In tissue cultures, PUVA is a potent mutagen (*3, 4*). In animals, it is carcinogenic and can produce both melanoma and nonmelanoma skin cancers [(*5*); Epstein JH: Personal communication]. Concern about the carcinogenic risks of PUVA, as well as its immunologic, ocular, and skin aging effects, has limited its long-term use for the treatment of psoriasis for patients with the most severe disease, older patients, or those for whom alternative therapies fail to benefit.

To date, investigators conducting 4 major studies have attempted to assess the long-term effects of PUVA in human beings: the 16-center prospective study of 1,380 patients in the United States (table 1), the Vienna–Innsbruck study, the Finnish study, and the PUVA-48 cooperative study. The interim results of the 16-center PUVA study that indicate a significant and dose-related increase in the risk of squamous cell carcinoma among patients treated with PUVA and a comparison of these results with those of the other 3 studies are presented.

ABBREVIATIONS: PUVA = psoralen plus UV radiation at 320–400 nm; UVB = UV radiation at 290–320 nm; J = joules.

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[4] I thank Mary Jean Moore for her editorial assistance.

MATERIALS AND METHODS

The methodologies, patient and control populations, and total exposures used in the 4 studies are compared, and possible explanations for differences in results are discussed.

The 16-center Psoralen Photochemotherapy Follow-up Study

Study Design

This is a prospective study of the long-term toxicity and efficacy of PUVA in 1,380 patients who began treatment as part of the oral methoxsalen photochemotherapy study in 1975–76 (*6*). Each year, patients are interviewed and given physical, ophthalmologic, and laboratory examinations. The interview and examinations document current health status, current psoriasis status and treatment, and the presence of cutaneous changes in this population. Particular attention is paid to the detection of new skin tumors. Of enrolled patients who are still living, 96% had an interview and 91% had a physical examination in 1980 or later.

Besides calculating the relative risk of developing skin cancer, the investigators conducting the study also determined the incidence of nonmelanoma skin cancers of different histologic types and examined the relationship between the development of individual tumors and PUVA dose, prior exposure to other psoriatic treatments presumed to be cutaneous carcinogens, and the site of tumor development.

Cohort characteristics.—At enrollment, the average age of patients was 43 years; 64% were male and 30% were of skin type I or II. Residents of all parts of the United States were included in the study. Past exposures to coal tar preparations, ionizing radiation, conventional UVB radiation, and methotrexate were frequent (*2*).

Controls used.—Participants compared the incidence of cutaneous carcinomas in patients treated with PUVA to that expected in people of similar age, sex, and geographic location in the general population (*7, 8*).

Exposure to psoralen photochemotherapy.—After 4 years of follow-up, most patients had had more than 100 treatments or more than 1,400 J/cm^2. (As of April 1982, most patients have been exposed to more than 1,600 J/cm^2.)

Results

As of December 1, 1980, after an average of 4 years of follow-up, 77 patients had developed a total of 145 nonmelanoma skin cancers; 35 patients had developed squamous cell carcinomas, 36 basal cell carcinomas, and 6 tumors of both types. There were 96 squamous cell carcinomas, compared with 49 basal cell carcinomas. The overall incidence of nonmelanoma skin cancer was similar to that first reported in this cohort in 1979; this incidence was 2.6 times higher than that expected (*3*). Follow-up for an additional year has

TABLE 1.—*Cooperating centers and investigators: Photochemotherapy Follow-up Study*

Institutions	Location	Investigators
Centers		
Baylor College of Medicine	Waco, Texas	Jarratt, M.
Beth Israel Hospital	Boston, Mass.	Arndt, K. A.
Columbia University College of Physicians and Surgeons	New York, N.Y.	Andrews, A. Harber, L. C.
Dartmouth Medical School	Hanover, N.H.	Baughman, R. D.
Duke University Medical Center	Durham, N.C.	Gilgor, R.
Massachusetts General Hospital	Boston, Mass.	Gonzalez, E.
Mayo Graduate School of Medicine	Rochester, Minn.	Muller, S.
Mt. Sinai Medical Center	New York, N.Y.	Frost, P.
Stanford University School of Medicine	Stanford, Calif.	Abel, E. Farber, E. M.
Temple University School of Medicine	Philadelphia, Pa.	Urbach, F.
University of California Medical School	San Francisco, Calif.	Cram, D. Epstein, J. H.
University of Miami Medical School	Miami, Fla.	Halprin, K.
University of Michigan Medical School	Ann Arbor, Mich.	Anderson, T. F. Voorhees, J.
University of Pennsylvania Hospitals	Philadelphia, Pa.	Petrozzi, J.
Washington Hospital Center	Washington, D.C.	Nigra, T. P.
Yale University School of Medicine	New Haven, Conn.	Braverman, I. M. Lucky, P.
Coordinating Center		
Harvard Medical School	Boston, Mass.	Fitzpatrick, T. B. Lange, R. H. Parrish, J. A. Stern, R. S.
Computer Medicine Laboratory		
Harvard Medical School	Boston, Mass.	Bleich, H. L. Melski, J. W. Slack, W. V.

indicated a continuation in these trends, with the development of new tumors in previously unaffected patients as well as in patients who had tumors previously.

The increase in the relative risk of nonmelanoma skin cancer in this population is principally due to the increased incidence of squamous cell carcinoma. After 4 years of follow-up, study patients were nine times more likely to develop these tumors than expected ($P<0.001$; chi-square test), whereas they were only twice as likely to develop basal cell carcinomas. Although more than 75% of basal cell carcinomas occurred on the head, neck, and upper extremities, less than 33% of squamous cell carcinomas occurred in these areas. Most of these latter tumors occurred on the lower extremities and trunk. Patients with squamous cell carcinomas had had a significantly higher number of photochemotherapy treatments before the development of their first cutaneous tumor than had patients who developed basal cell carcinomas ($P<0.005$; chi-square test).

Vienna-Innsbruck Study

Study Design

This prospective study (*9*) was confined to patients under active PUVA treatment who were monitored as part of their continuing therapy. The criteria for inclusion in the cohort were not documented, nor was any indication given that anyone made specific attempts to locate patients who were no longer under the care of the investigators. Duration of therapy and follow-up were identical for patients with and without tumors; both averaged 27 months.

Cohort characteristics.—The average age of patients was 45 years, and only 2% of patients were of skin type I. Total PUVA exposures, as well as previous exposures to other psoriatic treatments presumed to be cutaneous carcinogens (including arsenic, ionizing radiation, and cytotoxic drugs), were documented. Forty-one percent of the patients were considered to have had previous psoriatic treatments that were thought to be associated with a higher risk for skin cancer.

Controls used.—The incidence of skin cancers in patients treated with PUVA was compared with that expected in people of similar age and sex, as calculated from incidence data from the cancer registry of a Swiss canton.

Exposure to psoralen photochemotherapy.—With PUVA exposure documented in joules per square centimeter for all patients, the mean exposure determined was 660 J/cm^2.

Results

Eleven patients developed cutaneous carcinomas or actinic keratoses; 3 had squamous cell carcinomas, 1 had a basal cell carcinoma, 1 had a keratoacanthoma and an actinic keratosis, and 6 had a total of 12 actinic keratoses. This incidence was ten times higher than that expected. (It is important for the reader to note that cancer incidence rates for the control population used in this study are far lower

than those for the control populations used in the 2 United States studies.) Tumors occurred only among patients who had been exposed to other suspected cutaneous carcinogens before PUVA use. The authors characterized these patients as the high-risk group. Two of the 3 squamous cell carcinomas and 9 of the 13 actinic keratoses occurred on the lower extremities or trunk. Total UVA dose was higher in patients with keratoses or tumors than in patients without such lesions.

Finnish Study

Study Design

Of the 568 patients studied (*10*) who were first treated with PUVA in Helsinki prior to December 31, 1978, follow-up examinations were obtained for 525 (90%). The average duration of follow-up was 2.1 years, with a range of 1 to 3.6 years.

Cohort characteristics.—The mean age of patients was 43 years, 52% were male, and 92% were of skin type III or IV. Twenty-one percent reported that they had used arsenic treatments before PUVA, but only 3.6% reported prior treatment with ionizing radiation.

Controls used.—Investigators compared the incidence of skin cancer in patients treated with PUVA to that expected in a population matched for age and sex from the Finnish Cancer Registry and to the incidence in 1,033 patients with psoriasis who received hospital treatment for it in 1976. Whether tumors reported in these 1,033 patients represented new cases or were part of a cumulative incidence up to the time of examination was not specified.

Exposure to psoralen photochemotherapy.—At the time of follow-up, 82% of patients had received a total dose of less than 500 J/cm^2.

Results

Only 1 patient had a basal cell carcinoma, and it was believed to be a preexisting tumor. Two of 20 patients who had received more than 1,000 J/cm^2 developed bowenoid lesions. No increase in the relative risk of skin cancer in patients treated with PUVA was noted, compared with the control population derived from the Cancer Registry. The control population of psoriatic patients who had never been treated with PUVA had a higher incidence of skin cancer than those who had. No relationship between PUVA dose and the risk of skin cancer was shown, but bowenoid lesions were only detected in 2 patients who were among those with the highest total exposure to PUVA.

Psoralen Plus Ultraviolet Radiation-48 Cooperative Study

Study Design

Those (*11*) who conducted this cross-sectional study documented skin cancers that developed over a 4-year period in 631 patients who had been examined on one or more occasions as part of a multicenter PUVA study. The mean interval of prospective follow-up for these patients was not indicated.

Cohort characteristics.—The average age of patients was 46 years. Skin type was not recorded, nor was the cohort's exposure to carcinogenic treatments before PUVA use provided.

Controls used.—No controls were used.

Exposure to psoralen photochemotherapy.—Mean PUVA exposure for the group was slightly less than 1,000 J/cm^2.

Results

Ten patients developed cutaneous carcinomas, 3 had squamous cell carcinomas, and 7 had basal cell carcinomas. Three of these 10 patients were reported to have been treated previously with ionizing radiation. Mean total exposure to PUVA for patients who developed a tumor was higher than that for the study group as a whole.

DISCUSSION

All these studies documented that patients with psoriasis are at risk for developing skin cancer, but the investigators differed in their assessments of the degree of risk associated with PUVA therapy. Much of this discrepancy can be attributed to differences in 3 factors: study design, patient characteristics, and total PUVA exposure.

The United States 16-center follow-up study reported a significantly higher incidence of squamous cell carcinoma than that expected from incidence rates for a matched population and than that observed in the other 3 studies ($P<0.01$ for all comparisons, chi-square test). However, both the Austrian and the PUVA-48 cooperative studies were limited to patients who were continuing to receive care from the investigators, whereas all patients were followed prospectively in the 16-center study, regardless of their continuing care. Such an approach facilitates more complete ascertainment of tumors. Moreover, the length of follow-up is substantially greater in the 16-center study (a median of over 5 yr) than in the other 3 trials. Also important for one to note is that patients treated under both European protocols differed from those enrolled in the 16-center study. The Europeans were far less likely to have had prior exposures to ionizing radiation, long-term tar, or UVB phototherapy.

Perhaps the greatest difference between the 16-center study and the other trials was in the patients' total exposures to PUVA. After 4 years, most patients in the 16-center study had had at least 100 treatments. Only 10% of the Finnish patients had comparable exposure levels. The mean total UVA dose for patients in the Austrian study was less than one-half that for patients in the 16-center study. Among patients enrolled in the 16-center study who had not had substantial exposure to carcinogenic treatments prior to PUVA use and who had received fewer than 80 PUVA treatments, no increased risk of cutaneous carcinoma was observed (*12*). When skin type, previous exposure to carcinogenic treatments, and total PUVA exposure are considered, the results of the Austrian and Finnish trials are comparable to results of the 16-center study for patients with limited exposure to PUVA or to previous cancer treatments.

In all 4 studies, premalignant and malignant lesions were more frequent among patients with higher total exposure to PUVA. For example, the Finnish scientists noted the development of bowenoid lesions on areas normally protected from the sun in 2 of 20 patients who had been exposed to PUVA with more than 1,000 J/cm^2. Similar lesions were not

detected in patients with less exposure. In the Austrian study, patients with keratoses or tumors had a significantly higher total mean exposure to UVA than did patients without such lesions. All 3 squamous cell carcinomas reported in the PUVA-48 group occurred in patients who had received more than 1,300 J/cm^2, which is a dose substantially higher than that received by the study population as a whole. The 16-center study noted that patients with more than 80 PUVA treatments (the equivalent of 1,200 J/cm^2) were at highest risk for squamous cell carcinoma.

In 3 of the 4 studies, a higher incidence of skin tumors was detected among patients with exposure to carcinogenic treatments before PUVA was used. These observations and those in other patients who developed tumors during PUVA treatment (including 1 who spontaneously regressed after PUVA treatment was discontinued) have led to the hypothesis that PUVA may be acting as a pseudopromoter or co-carcinogen, thus allowing the expression of preexistent malignant foci that would have otherwise been controlled (*13*). This hypothesis is also supported by the observation that multiple squamous cell carcinomas developed in patients with mycosis fungoides who received PUVA therapy after exposure to electron beam or topical nitrogen mustard (*14*).

Although the investigators who participated and contributed their data vary widely in their estimates of the magnitude of the risk of cutaneous cancer associated with PUVA, all identified a subset of patients who developed an increased number of atypical keratinocytic proliferative lesions in unusual anatomic sites after substantial PUVA exposure. The pathologic diagnoses for these lesions range from actinic keratosis to squamous cell carcinoma. Whether the increased frequency of these lesions is related to the individual patient's ability to repair PUVA-induced DNA damage, PUVA-induced immunologic alterations, or its action as a co-carcinogen in patients with prior exposure to other carcinogens has not been established (*13-16*). Determination of the mechanisms by which prolonged PUVA exposure increases the risk of nonmelanoma skin cancer would add to our knowledge about the pathogenesis of carcinoma and should increase our ability to select patients for PUVA therapy who are at lowest risk of developing such tumors.

In animals, PUVA initiates cutaneous carcinomas (*5*). A high incidence of dysplastic epidermal keratinocytes has been observed in patients treated with PUVA (*17*). These dysplastic cells may persist after cessation of therapy. As past experience with superficial ionizing radiation to the skin indicates, any determination of the full extent of PUVA activity as a primary carcinogen may require 10 years or more.

Of greater clinical importance than the incidence of cutaneous tumors in patients treated with PUVA is the natural history of these tumors. After 5 years, squamous cell carcinomas that have occurred in patients treated with PUVA have rarely if ever metastasized. In this way, they are like squamous cell carcinomas induced by sun exposure in patients who have never received PUVA. However, long-term surveillance is required for physicians to be assured that 1) tumors associated with PUVA exposure will continue to act in this fashion and 2) tumors which may develop after a longer latency following first PUVA exposure will also be unlikely to metastasize.

No randomized, long-term controlled study of PUVA toxicity now exists, and it is unlikely that such a study will ever be done. However, the continued, careful, long-term prospective study of the 16-center cohort, who have already been followed for more than 5 years, should allow a comprehensive evaluation of patient characteristics associated with the risk of developing tumors after PUVA use. Continued study should also help us determine the relationship between PUVA dose and the risk of tumor development and define the ultimate carcinogenic risk of such therapy.

Most importantly, continued study of a carefully followed cohort is the only practical means by which we can assess whether PUVA exposure increases the risk of biologically aggressive squamous cell carcinomas, malignant melanomas, or lymphoreticular neoplasms. Although it has been suggested that PUVA may have these more serious side effects and case reports of the occurrence of melanoma and leukemia in patients treated with it have been published, data from the 16-center study have not demonstrated any significant relationship between PUVA exposure and an increased risk of such tumors (*12, 18-21*).

REFERENCES

(*1*) PARRISH JA, FITZPATRICK TB, TANENBAUM L, et al: Photochemotherapy of psoriasis with oral methoxsalen and long-wave ultraviolet light. N Engl J Med 291:1207-1211, 1974

(*2*) STERN RS, THIBODEAU LA, KLEINERMAN RA, et al: Effect of oral methoxsalen photochemotherapy on cost of treatment for psoriasis. JAMA 245:1913-1918, 1981

(*3*) CARTER DM, WOLFF K, SCHNEDL W: 8-Methoxypsoralen and UVA promote sister-chromatid exchanges. J Invest Dermatol 67:548-551, 1976

(*4*) ASHWOOD-SMITH MJ, GRANT EL, HEDDLE JA, et al: Chromosome damage in Chinese hamster cell sensitized to near ultraviolet light by psoralen and angelicin. Mutat Res 43:377-385, 1977

(*5*) GRIFFIN AC, HAKIM RE, KNOX J: The wavelength effect upon erythemal and carcinogenic response in psoralen treated mice. J Invest Dermatol 31:289-295, 1958

(*6*) MELSKI JW, TANENBAUM L, PARRISH JA, et al: Oral methoxsalen photochemotherapy for the treatment of psoriasis: A cooperative clinical trial. J Invest Dermatol 68:328-335, 1977

(*7*) SCOTTO J, FEARS TR, GORI G: Measurements from Ultraviolet Meters at Ten Locations in the United States During 1914 and Comparisons With Recent Skin Cancer Data. DHEW Publ (NIH) 76-1029. Washington, D.C.: U.S. Govt Print Off, 1975

(*8*) SCOTTO J, FEARS TR, FRAUMENI JF: Incidence of Nonmelanoma Skin Cancer in the United States. DHHS Publ (NIH) 82-2433. Washington, D.C.: U.S. Govt Print Off, 1982

(*9*) HÖNIGSMANN H, WOLFF K, GSCHNAIT F, et al: Keratoses and nonmelanoma skin tumors in long-term photochemotherapy (PUVA). J Am Acad Dermatol 3:406-414, 1980

(*10*) LASSUS A, REUNALA T, IDÄNPÄÄ-HEIKKILÄ J, et al: PUVA treatment and skin cancer: A follow-up study. Acta Derm Venereol (Stockh) 61:141-145, 1981

(*11*) ROENIGK HH JR, CARO WA: Skin cancer in the PUVA-48 cooperative study. J Am Acad Dermatol 4:319-324, 1981

(*12*) STERN RS, ZIERLER S, PARRISH JA: Psoriasis and the risk of skin cancer. J Invest Dermatol 78:147-149, 1982

(*13*) STRAUSS GH, BRIDGES BA, GREAVES M, et al: Inhibition of

delayed hypersensitivity reaction in skin (DNCB test) by 8-methoxypsoralen photochemotherapy. Possible basis for pseudo-promoting action in skin carcinogenesis? Lancet 2:556–559, 1980

(*14*) VERDICH J: Squamous cell carcinoma. Occurrence in mycosis fungoides treated with psoralens plus long-wave ultraviolet radiation. Arch Dermatol 115:1338–1339, 1979

(*15*) BRIDGES BA, STRAUSS GH: Possible hazards of photochemotherapy for psoriasis. Nature 283:523–524, 1980

(*16*) MORISON WL, PARRISH JA, MOSCICKI R, et al: Abnormal lymphocyte function following long-term PUVA therapy for psoriasis. J Invest Dermatol 76:303, 1981

(*17*) COX AJ, ABEL EA: Epidermal dystrophy. Occurrence after psoriasis therapy with psoralen and long-wave ultraviolet light. Arch Dermatol 115:567–570, 1979

(*18*) DURKIN W, SUN N, LINK J, et al: Melanoma in a patient treated for psoriasis. South Med J 71:732–733, 1978

(*19*) HANSEN NE: Development of acute myeloid leukemia in a patient with psoriasis treated with oral 8-methoxypsoralen and long-wave ultraviolet light. Scand J Haematol 22: 57–60, 1979

(*20*) International Commission for Protection Against Environmental Mutagens and Carcinogens: Do treatments available for psoriasis carry a genetic or carcinogenic risk? Mutat Res 86:279–304, 1981

(*21*) WAGNER J, MANTHORPE R, PHILIP P, et al: Preleukemia (haemopoietic dysplasia) developing in a patient with psoriasis treated with 8-methoxypsoralen and ultraviolet light (PUVA treatment). Scand J Haematol 21:299–307, 1978

DISCUSSION

K. Halprin: Dr. Stern's paper on carcinogenicity of PUVA photochemotherapy in humans is open for discussion.

J. E. Hearst: Dr. Stern, you mentioned that there is a seventeenfold increase in risk of cancer in patients with skin types I and II in the general population. Is that a comparison with all Caucasians or with those in the general population having skin types I and II?

R. S. Stern: That is compared with all Caucasians, which is why I said it is a completely predictable kind of increase.

Unidentified Participant: Have any of the squamous cell carcinomas metastasized?

Stern: One patient with squamous cell carcinoma has had a metastasis for certain, but I believe it unlikely that it was due to PUVA, certainly not to PUVA alone. The primary tumor was in the buttock or perianal area, which probably did not get much exposure to PUVA therapy. Also, this person was previously exposed to ionizing radiation. So, yes, metastasis has occurred, but I believe treatment with PUVA was not the cause.

Halprin: You must admit that the number of patients in this study is large.

Stern: That is right.

Halprin: They are not being seen by doctors in their private offices. We reported 29 patients with carcinomas not treated with PUVA but followed over a 5-year period. Fourteen of those occurred in the covered areas; 13 of which were squamous cell lesions. The age distribution was the same as you have shown. Basal cell carcinomas had developed in people in their 60s, and squamous cell lesions peaked in those in their 50s.

Over 300 of your patients received X-ray therapy; I would not be surprised if all 300 had developed squamous cell carcinomas. You reported only 87 patients now have tumors; I do not understand why the 300 do not. The incidence of cancers in these people does not surprise me.

Stern: I think a few factors account for this number. First of all, we have about 300 people who are on record for having received ionizing radiation. Clearly, not necessarily every one of those patients actually had ionizing radiation because it is historical. Secondly, the dose and extent of body area treated obviously varied substantially. Some people may have had minute and ineffective exposures to ionizing radiation.

Given the relatively widespread use of ionizing radiation for psoriasis in the past, if your hypothesis held true and nearly everyone treated with ionizing radiation were to get a squamous cell lesion, I think our failure to note these tumors before PUVA says something about all dermatologists as observers from the beginning of the ionizing radiation era up until 1979.

The observation in our population is that, although about one-quarter of the population reports having received ionizing radiation, one-half the patients with tumors and more than one-half of those with squamous cell lesions are patients who have no previous history of treatment with ionizing radiation, many of whom we are quite certain did not. Furthermore, within this latter group are young people who developed multiple squamous cell carcinomas and who did not show substantial actinic damage on their pre-PUVA physicals. One could attribute these observations to a number of factors. The increase may be due to ionizing radiation. If ionizing radiation alone were the only factor, one would expect that the ratio of squamous cell to basal cell carcinomas in these 2 groups should be the same. I believe that these data argue strongly for a dose dependency between PUVA and an increasing risk of squamous cell carcinoma among those patients who are predisposed to it because of exposures they had before PUVA treatment.

An additional mechanism that has been suggested by others may be tumors developed in the small percentage of patients who are particularly sensitive to DNA or other damage caused by PUVA and are therefore likely to produce squamous cell carcinomas when exposed to PUVA. I think these data clearly point to some relationship.

Halprin: Let us look at the lag-time data on the animals. Once you are past the lag time, the incidence of carcinomas rises dramatically. We are now past that lag period for the carcinomas that are occurring because of previous ionizing radiation.

After that time, the incidence rises steeply once it starts. The interval here is longer for the 80-plus treatments, I would suppose.

Stern: I standardized the interval for each of the 3 groups. You will note that the squamous cells come preferentially from the greater-than-140-treatment group. The mean interval from the first treatment to either the third follow-up examination for patients without tumors or to the development of a first tumor is not significantly different among these 3 groups.

Halprin: How large is the patient population?

Stern: I will have to do a little calculating. Approximately

25 patients are developing squamous cell lesions more than 3 years but less than 5 years after receiving PUVA therapy. Approximately 20 patients developed basal cell carcinomas in this time, and the remaining group is comprised of more than 1,000 patients who have been followed more than 3 years. This analysis was restricted to patients over 30. Does that answer your question?

Halprin: Yes.

G. Lazarus: Is the effect of ionizing radiation independent of the port of entry?

Stern: Again, as I have tried to emphasize, my data do not permit pinpointing sites. That is why I tend to speak in general terms and not mechanistic terms. I do not consider my data sufficiently reliable to allow me to look at the relationship between the area irradiated and site of tumor development. I cannot answer that question.

Lazarus: Would you care to make an educated guess?

Stern: I really would not. There are some events that I feel confident about, like the development of a tumor; but I have little confidence in others that may be subject to substantial bias.

Lazarus: When you started talking about ionizing radiation or routine forms of irradiation, e.g., chest X-rays, etc., as I recollect, you did not indicate those sources as being causative.

Stern: I am referring only to therapeutic ionizing radiation for the treatment of psoriasis. The question participants were asked was: "Have you ever had grenz rays or ionizing radiation for the treatment of your psoriasis?"

Lazarus: You actually used the word "diagnostic."

Stern: That should have been "therapeutic." I am sorry.

J. H. Epstein: Have you any information on melanoma formation?

Stern: Yes. We had 2 patients whose melanomas were documented by biopsies, 1 of which I am personally confident had no relation to PUVA, in that it was noted on the patient's foot early in the course of therapy. It was probably a preexisting lesion. The other patient had an extraordinarily superficial melanoma that was only 0.3 mm, level II of an unclassifiable type. He had 296 treatments before he developed this tumor. Clinicians who have been following him quite closely believe that this did not arise in a lesion present before PUVA, but rather arose in a pigmented lesion that developed after treatment began.

We observed a couple of other lesions that were reviewed and called dysplastic lesions or lentigo maligna but not melanomas.

Epstein: We produced our first melanoma in the mouse with topical psoralen, as of now (March 1982). I do not know how many will develop by the end of the experiment.

T. B. Fitzpatrick: I think that Dr. Stern and the group have done an excellent job in providing a data base that we can refer to when we read about isolated anecdotal incidents of toxic effects from PUVA. We do not have any such data for UVB; let us hope a data base will be available in the future.

We also have to apply the same rigid criteria in interpreting case reports. We know that patients with xeroderma pigmentosum develop skin cancers almost constantly, and it would be difficult to document an increased number of lesions. This is an unproved association and should not be regarded as evidence for photocarcinogenicity of psoralen.

Stern: We looked carefully at causes of death and incidences of systemic carcinoma in our population. As I mentioned, 90 of 1,380 people died, which is essentially what one would expect in a population of this age distribution followed for the length of time. We compared the cancer incidence with Connecticut Cancer Registry data, and our incidence data are not significantly different from what we would expect based on those of the Registry.

Furthermore, we studied the proportional incidence of various types of cancers, which is perhaps a better figure to look at than absolute incidence, and we find no difference in the relative incidence of lymphoreticular tumors or other types of neoplasms that one might be more concerned about as being caused by PUVA in relation to other types that would less likely develop as a result of such therapy. From our data, we have no evidence that any significant or substantial increase in mortality is due to PUVA treatment given to patients with noncutaneous tumors.

Halprin: I would like to have you consider the enormous difference between the European and American experience. This difference is not just in regard to PUVA itself. They have an enormous difference in their background rates of skin cancer. The figure we accept here is the incidence rate per year. In all the American studies, the incidence of skin cancer per year in the general population is 0.4%. That is five times the skin cancer rate that they use in the European studies (0.08%/yr). We are dealing with genetically different people as well as the fact that they did not use X-ray in their countries as extensively as we have here.

D. M. Carter: Could you comment on what you mean by a background genetic difference between the European and the American populations?

Halprin: I think our population may be skewed toward the Celtics or some other cancer-prone type who are probably twenty times more sensitive than light-skinned Europeans and Scandinavians.

Risk of Skin Tumors in Psoralen- and Ultraviolet A-treated Patients[1]

Tilo Henseler *and* Enno Christophers[2]

ABSTRACT—From 1973 to 1981, the clinical data of 1,136 patients with psoriasis and another 1,210 with various skin tumors were accessioned with the aid of computerized data files. Skin tumors and psoriasis occurred in 48 patients. After psoralen plus 320–400 nm UV (PUVA) therapy for psoriasis was introduced in 1976, 381 patients with this disease were treated. Follow-up data revealed no change in the tumor incidence rate after this treatment began. Age-related calculations of the relative probabilities for the presence of skin cancer in patients with psoriasis after PUVA revealed a grossly normal pattern (which appeared to be unaffected by PUVA). We observed a skin tumor in 2 patients 5 years after PUVA therapy. — Natl Cancer Inst Monogr 66: 217–219, 1984.

Photochemotherapy of psoriasis and other dermatoses has been successful during the last 7 to 8 years. An adequate dose of PUVA is known to react with DNA, and interstrand cross-links are formed that may not be repaired. In view of the fact that DNA damage may initiate cancer, PUVA-induced skin changes have attracted considerable attention clinically and histologically.

In recent years, 4 major surveys have been reported on the incidence of skin cancer in PUVA-treated patients (*1–4*). In each survey, incidence ranged from 0.45 to 2.2% of the total number of patients. Multiple tumors present in a single patient were included. The investigators also attempted to recognize subgroups who are more prone to develop cancers, i.e., those patients who had undergone radiation therapy (unspecified) or previous treatment with arsenic (unspecified). Unfortunately, lack of data prevented comparison with controls on the incidence of skin tumors in patients with psoriasis who did not receive PUVA. Therefore, these data have been interpreted with reservations (*5*).

In our study, we tried to analyze longitudinally the incidence of single cases of skin cancer and psoriasis and that of both in the same patient before and after the advent of PUVA. Although the length of observation may not be sufficient, the present data do not support the concept of PUVA as a potent carcinogen.

MATERIALS AND METHODS

The computerized files of the Department of Dermatology, University of Kiel, contain data relevant to the clinical course, laboratory findings, and results of treatment in 10,260 hospitalized patients from 1973 to 1981. From these files, data pertinent to diagnosed psoriasis, skin neoplasms, and both (by type, age, sex, year) were collected in collaboration with the Department of Documentation and Statistics, also at the University. Every patient under consideration is statistically counted once on the date of his/her first treatment at the department.

After PUVA therapy was introduced in 1976 and administered as described in (*6*), all of the 381 patients selected were hospitalized for the first treatment, so that the results and any side effects could be closely monitored. The patients reported here suffered from extensive body involvement (>50% body surface affected). They were carefully examined for the presence of skin tumors, all of which were excised and examined by routine histology if malignancy was suspected.

RESULTS

Figure 1 shows the age distribution of the patients with psoriasis, melanoma, and basal cell carcinoma at the time the diagnosis was made here. Nearly one-half the patients with psoriasis are younger than 40 years, with a peak incidence around 22 years. For the patients with all forms of melanoma, the peak diagnostic incidence occurs at approximately 50 years of age, the maximum rate of those with basal cell carcinoma was observed when the patients were 75 years of age. Spot calculations for the years 1974 and 1980 revealed no differences, which indicated the absence of major changes in age distribution. These curves demonstrate the proportional distribution of the diagnostic groups under discussion and emphasize the early onset of psoriasis compared with skin neoplasms.

In table 1, the number of patients hospitalized for psoriasis or skin tumors, or both, are listed yearly from 1973 to 1981. The number of patients with psoriasis rose in 1976 and 1977 after PUVA was introduced. Thereafter, inpatient treatment steadily decreased. Increasing numbers of patients with skin tumors were treated since 1976, and this number nearly tripled in 6 years. Contrary to this, no change in the simultaneous occurrence of psoriasis and skin cancer was noted. Patients with both diseases were seen at a rate of 1 to 7 per year (table 1) for a total of 48 skin tumors tabulated in 1,136 patients with psoriasis during the period from 1973 to 1981.

Table 2 shows the calculated probabilities for the presence of either psoriasis or skin cancer, or both. The results, based on the number of hospitalized patients per year, show that the rates are almost steadily decreasing for psoriasis and doubling for cancer. Furthermore, the probability of having both diseases remains constant. Interestingly, the calculated incidence rate of simultaneously having both diseases is 0.87% (table 2). If one assumes that psoriasis and skin cancer are independent events, the calculated age-adjusted incidence rate for both diseases is much higher than the incidence rate observed in our data (0.47%, table 2). This figure

ABBREVIATION: PUVA = psoralen and UV radiation at 320–400 nm.

[1] Presented at the Conference on Photobiologic, Toxicologic, and Pharmacologic Aspects of Psoralens held in Research Triangle Park, North Carolina, March 1–3, 1982.

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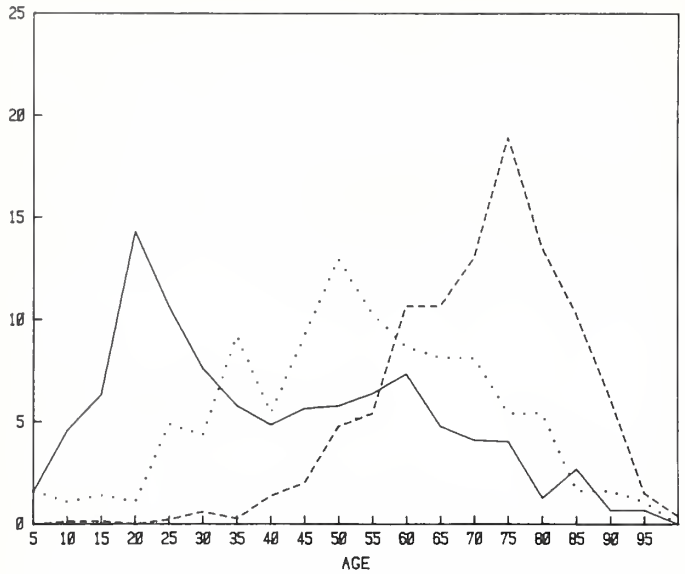

FIGURE 1.—Age distribution of patients with psoriasis, melanoma, and basal cell carcinoma when the diagnosis was first made. Incidence is calculated as percent of the total groups (*ordinate*). *Solid line* = psoriasis; *dotted line* = melanoma; *dashed line* = basal cell carcinoma.

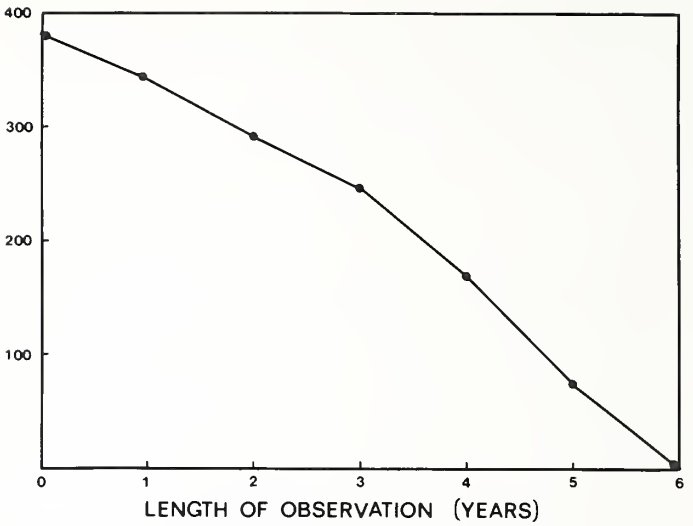

FIGURE 2.—Length of observation in 381 PUVA-treated patients. Data given on the *ordinate* are absolute numbers of patients under control for the time depicted in the *abscissa*, e.g., 169 patients were clinically observed for 4 yr.

supports previous observations on the generally low incidence of skin cancer in patients with psoriasis (*7*).

Inasmuch as the appearance of PUVA-related skin cancer may depend on time, the number of years the PUVA-treated patients were under observation were calculated. Figure 2 illustrates that the number of patients under control after PUVA treatment decreases almost linearly over 6 years. Consequently, a considerable number of patients (169) were observed for longer than 4 years after the initial and maintenance treatment.

Among the psoriasis-plus-cancer patients, psoriasis was diagnosed first in 7 patients; their tumors appeared later (table 3). Skin cancer with psoriasis (usually mild) was diagnosed simultaneously in 41 patients. The numbers listed in table 3 indicate some yearly fluctuation. However, no gross changes after the onset of PUVA therapy become overt. A preponderance of the patients in the older age groups (70–80 yr) is seen reflecting an undisturbed picture of tumor incidence (table 4).

Tumors were seen in 3 PUVA-treated patients in 1976, 1980, and 1981 (table 3). Close examination revealed that the patient seen in 1976, who had been taking arsenic, had a basal cell carcinoma when PUVA was started. The data on the other 2 patients are listed in table 5. Both had psoriasis for more than 40 years and were treated with the whole spectrum of therapeutic modalities including coal tar and 280–320 nm UV. The location of the tumors (1 basal cell carcinoma, 1 squamous cell carcinoma) is suggestive of being related to PUVA therapy.

DISCUSSION

The data presented are based on continuous listing of patient-related information, so that trends can be visualized, especially with the advent of new therapeutic modalities. Our figures are influenced by a change in medical interests taking place in 1976 and the following years as the result of advances in hospital management of patients.

In figure 1, the age distribution of the patients with diagnoses of psoriasis, melanoma, and basal cell carcinoma reveals marked differences. These differences support the notion that a comparison of incidence rates between 2 diagnostic groups needs age-related re-calculation, the results of which are shown in table 2. A steady increase in the number of cancer patients began in 1976. These figures include patients with malignant melanoma; e.g., in 1980 the annual number of hospitalized melanoma patients was 83. However, in none of the PUVA patients was malignant melanoma detected.

Contrary to cancer, the yearly incidence of treated psoriasis shows a sharp rise in 1976 and 1977 and levels off thereafter; this coincides with the introduction of PUVA in our department in 1976. Furthermore, nearly all patients with extensive body involvement received PUVA treatment

TABLE 1.—*Number of patients treated for psoriasis, skin tumors, and both*

Diagnosis	1973	1974	1975	1976	1977	1978	1979	1980	1981	Total
Psoriasis	157	132	98	120	186	134	121	106	87	1,136
Psoriasis, with PUVA treatment				75	94	76	47	51	38	381
Skin tumors, including melanomas	74	78	75	126	140	156	156	199	201	1,210
No. of skin tumors and psoriasis	7	4	2	7	7	7	1	6	7	48

TABLE 2.—*Age-adjusted probability of the presence of psoriasis, skin tumors, and both before and after PUVA*

Yr	Psoriasis	Skin tumors	Psoriasis plus skin tumors	Calculated probability
1973	16.7	7.9	0.75	0.88
1974	14.2	8.4	0.43	0.79
1975	10.9	8.3	0.22	0.61
1976	10.8	11.4	0.63	0.82
1977	13.9	11.6	0.52	1.07
1978	11.1	13.0	0.58	0.96
1979	9.7	12.9	0.08	0.83
1980	7.8	15.3	0.46	0.73
1981	6.7	15.6	0.54	0.70
Total	11.1	11.8	0.47	0.87

in the hospital and were later put on intermittent PUVA therapy as outpatients. The decreasing values for psoriasis in 1978 and later probably reflect a decreasing incidence of severe untreated psoriasis. This finding correlates with an increasing number of outpatients receiving PUVA for psoriasis since then (data not shown).

This result is in line with the decreasing calculated probability of a person's being treated for psoriasis beginning in 1978. On the other hand, the probability of being hospitalized for the treatment of skin cancer (including melanomas) increases with the introduction of PUVA so that, theoretically, PUVA could be implicated as a causal factor. However, as the probability of having both psoriasis and cancer remains constant, external causes, e.g., PUVA, do not appear to be influential. Moreover, data on the outpatients excluded any changes in tumor incidence other than those reported here. On the basis of these findings, we believe that, for the time of our observation, PUVA does not appear to be a significant causal factor in skin cancer.

The patients under observation represented a homogeneous population sample from a restricted geographic region. In addition, this study was conducted longitudinally over nearly a decade. Therefore, changes in cancer incidence rates are representative and not influenced by factors

TABLE 3.—*Occurrence of skin tumors in psoriasis patients*

Yr	No. of patients with: Tumors and psoriasis simultaneously	Tumors after onset of psoriasis
1973	4	3
1974	3	1
1975	2	
1976	6	1
1977	7	
1978	7	
1979	1	
1980	5	1
1981	6	1

TABLE 4.—*No. of skin tumors in relation to age of patient*

Age, yr	Tumors after onset of psoriasis	Tumors plus psoriasis
30		
40		2
50	1	3
60	1	7
70	2	13
80	3	12
90		

TABLE 5.—*Clinical data of 2 patients with neoplasms after PUVA therapy*[a]

Patient No.	Age, yr	Age at onset of psoriasis, yr	Type of tumor	UVA, J/cm^2	Area
1	64	42	Basal cell carcinoma	1,354	Abdomen
2	77	63	Squamous cell carcinoma	1,039	Gluteal region

[a] No risk factors were noted. Both patients received PUVA for 5 yr.

involved in multicenter studies. The patient files included those patients who had received arsenic or localized ionizing radiation for various reasons, a group at risk to develop cancer after PUVA (*3*). Although we paid attention to this factor in our present study, no information can be supplied that supports an increasing susceptibility to PUVA-induced skin cancer in this subgroup.

REFERENCES

(*1*) STERN RS, THIBODEAU LA, KLEINERMAN RA, et al: Risk of cutaneous carcinoma in patients treated with oral methoxsalen photochemotherapy for psoriasis. N Engl J Med 300:809–813, 1979

(*2*) HÖNIGSMANN H, WOLFF K, GSCHNAIT F, et al: Keratoses and nonmelanoma skin tumors in long-term photochemotherapy (PUVA). J Am Acad Dermatol 3:406–414, 1980

(*3*) ROENIGK HH JR, CARO WA: Skin cancer in the PUVA–48 cooperative study. J Am Acad Dermatol 3:319–324, 1981

(*4*) HÖNIGSMANN H: Langzeiteffekte der Photochemotherapie (PUVA). *In* Fortschritt und Fortbildung in der Medizin, Jahrbuch 1981/82 (Odenbach E, ed). Köln: Deutscher Ärzte-Verlag, 1981, pp 371–377

(*5*) PEMBROKE AC, HEHIR ME, DUVIVIER AW, et al: Photochemotherapy and risk of skin cancer. Lancet 1:1299, 1979

(*6*) HENSELER T, HÖNIGSMANN H, WOLFF K, et al: Oral 8-methoxypsoralen photochemotherapy of psoriasis. The European PUVA Study: A cooperative study among 18 European centres. Lancet 1:853–857, 1981

(*7*) SHUSTER S, CHAPMAN PH, RAWLINS MD: Psoriasis and cancer. Br Med J 1:941–942, 1979

Effects of Psoralens Plus Ultraviolet Radiation On Human Lymphoid Cells in Vitro[1]

Kenneth H. Kraemer[2,3] *and* Haywood L. Waters[2]

ABSTRACT—Photochemotherapy with oral 8-methoxypsoralen (8-MOP) plus long wavelength UV radiation (UVA) has been shown to affect lymphoid cells circulating through the skin. An in vitro assay was developed to mimic some of the therapeutic parameters of 8-MOP concentration and UVA exposure estimated to impinge upon lymphoid cells. In vitro treatment with these presumed therapeutic levels of 8-MOP plus UVA induced a level of inhibition of lymphoid cell DNA synthesis similar to that observed in vivo. Furthermore, the DNA synthesis inhibition was associated with DNA interstrand cross-link induction, reduced cell survival, and impaired immune reactivity. This assay predicts that such effects would be induced in vivo. A lymphoblastoid cell line from a patient with Cockayne's syndrome was shown to be hypersensitive to killing by radiation (280–320 nm) from a fluorescent sunlamp (UVB) but to have normal survival after treatment with 8-MOP plus UVA. Thus there is at least one major UVB recovery pathway in human cells that is different from the recovery pathway for 8-MOP plus UVA damage and leads us to believe that combined treatment with UVB and 8-MOP plus UVA may have a greater effect than either treatment alone. — Natl Cancer Inst Monogr 66: 221–223, 1984.

Psoralen photochemotherapy combining oral 8-MOP with UVA (PUVA) is under investigation for treatment of human skin diseases, such as psoriasis and mycosis fungoides (*1–4*). In oral 8-MOP photochemotherapy, the skin is thought to be the primary treatment target, but lymphoid cells may also be affected. Inasmuch as the 8-MOP is taken orally, the drug appears in the blood, and the circulation of blood through the dermis is substantial (*5*). In disease conditions, this circulation may be increased and, in addition, leukocytes may accumulate in the dermis. Because UV radiation is only partially filtered by the epidermis (*6–8*), these psoralen-bathed dermal lymphoid cells would be exposed to UVA. Thus the lymphoid cells may also be targets of PUVA therapy.

To test this hypothesis, we conducted an experiment measuring DNA synthesis in circulating leukocytes of patients with widespread psoriasis who were under treatment with 8-MOP photochemotherapy (*9*). We expected that if the leukocytes were receiving substantial 8-MOP plus UVA, DNA synthesis would be inhibited. In the experiment, 7 of 13 treated patients demonstrated a statistically significant reduction in leukocyte incorporation of [^{3}H]dThd immediately after UVA in comparison to incorporation before UVA. The leukocyte DNA synthesis, reduced by 40–60% in these patients, was evidence that photochemotherapy affected circulating lymphoid cells in vivo. More recently, others have shown similar effects on DNA synthesis and on immune reactivity in vivo (*10–16*).

To determine whether the observed inhibition of lymphoid cell DNA synthesis could be a direct effect of 8-MOP plus UVA, we developed an in vitro assay to reproduce some of the features of in vivo therapy (*17–21*). The assay system consists of suspension cultures of fresh lymphocytes or Epstein-Barr virus-transformed human lymphocytes (lymphoblastoid cell lines) which are placed over a plate glass filter above 4 parallel clinical UVA fluorescent lamps. The plate glass serves both as a support for the culture flasks and as an optical filter to mimic the UV radiation-filtering effect of the epidermis. We controlled the temperature at 37° C and kept the cells in suspension by shaking the plate glass. This assay system permits quantitation and simultaneous correlation of multiple biologic and physical effects of photochemotherapy on human lymphoid cells.

We used this assay system to measure DNA synthesis in lymphocytes and lymphoblastoid cells after their in vitro treatment with 8-MOP at therapeutic concentrations of 0.01 to 1.0 μg/ml followed by UVA (*21*). Dose-dependent decreases in [^{3}H]dThd incorporation were found with both types of lymphoid cells (fig. 1). At 8-MOP concentrations of 0 and 0.1 μg/ml, the lymphoblastoid cell and lymphocyte curves were not significantly different ($P>0.2$). At 1 μg 8-MOP/ml, the lymphoblastoid cell curve was significantly different ($P<0.001$) from that of the lymphocytes. We noted that DNA synthesis was more sensitive to changes in UVA exposure than to changes in 8-MOP concentration. For the lymphoblastoid cells, in the range of doses studied, we found that increasing the UVA exposure tenfold resulted in a 30% increase in inhibition of DNA synthesis, whereas increasing the drug concentration similarly resulted in 20% additional inhibition.

Lymphoid cells have been estimated to receive 0.8 to 4% of the skin surface dose during photochemotherapy [*see* (*9*) and (*21*) for discussion of this estimate]. Thus typical therapeutic skin surface exposures would be estimated to deliver 1,000 to 7,000 joules UVA/m^2 to circulating lymphoid cells. These in vitro exposures reduced dThd incorporation in cells pretreated with 8-MOP by 40–60%, an extent of inhibition similar to that observed in vivo. Hence the observed reduction in DNA synthesis in vivo may be a direct effect of UVA acting on 8-MOP-sensitized circulating leukocytes.

Using the in vitro assay, we were able to demonstrate that

ABBREVIATIONS: 8-MOP = 8-methoxypsoralen; UVA = UV radiation at 320–400 nm; PUVA = 8-MOP plus UVA; dThd = thymidine; UVC = UV radiation at 254 nm; UVB = UV radiation at 280–320 nm.

[1] Presented at the Conference on Photobiologic, Toxicologic, and Pharmacologic Aspects of Psoralens held in Research Triangle Park, North Carolina, March 1–3, 1982.

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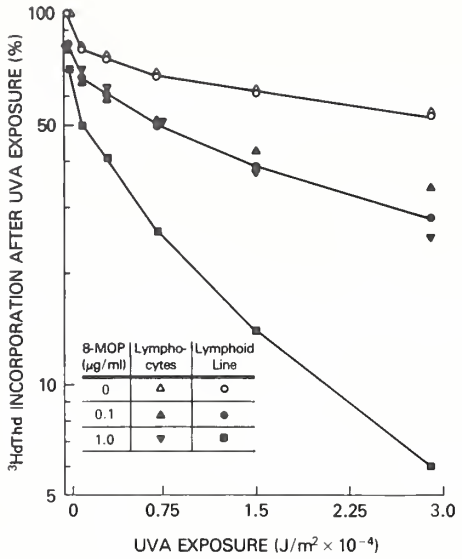

FIGURE 1.—Inhibition of DNA synthesis by 8-MOP plus UVA in lymphocytes and lymphoblastoid cell lines. Fresh lymphocytes from normal donors or lymphoblastoid cell lines (E1 and GM 1953) were suspended in salt solution and treated with 8-MOP followed by exposure to plate glass-filtered PUVA lamps. Incorporation of [^{3}H]dThd was measured during the first 2 hr after UVA exposure. Data presented are the estimated mean relative amounts of [^{3}H]dThd incorporation from a large series of experiments [modified from (*17*)]. J = joule(s).

similar doses of 8-MOP plus UVA induced DNA interstrand cross-links (*20, 22*) and reduced cell survival (*17, 20*). This therapy induced inhibition of DNA synthesis which did not affect cell survival when 60–100% of [^{3}H]dThd incorporation remained. However, greater inhibition of DNA synthesis was highly correlated with decreased cell survival (*21*). Also the degree of correlation was high between reduced cell survival and the relative number of cross-links induced by different combinations of 8-MOP plus UVA (*20*). Thus the in vitro model predicts that 8-MOP photochemotherapy which results in greater than 40% inhibition of DNA synthesis in circulating leukocytes would induce DNA cross-links in these cells and diminish cell survival.

An aspect of leukocyte function requiring healthy cells is immune reactivity. As a model for immune reactivity, we measured the mixed leukocyte reaction in fresh leukocytes from normal donors which were exposed to PUVA in vitro (*23*). The treated cells showed diminished stimulation ability as well as impaired proliferation. An unexpected observation was that the UVA alone, in the absence of 8-MOP, caused a significant inhibition of mixed leukocyte reaction stimulation ability. Others (*11–15*) have found clinical evidence of diminished immune reactivity in psoriasis patients treated with PUVA. The in vitro experiments suggest that at least some of the impaired immune reactivity may be a direct effect of 8-MOP plus UVA on circulating leukocytes.

Recent clinical discussion has focused on the possibility of combined therapy with PUVA plus an additional modality. The rationale for such a program would be that by choosing therapies with different mechanisms of action, one might produce synergistic beneficial effects with less toxicity than by using either treatment alone. The use of an in vitro assay involving cells from patients with cellular hypersensitivity to killing by physical or chemical agents may provide evidence for various mechanisms of actions of different therapies. If cells are defective in the ability to recover from 1 agent but have a normal response to treatment by the other agent, there must be at least one recovery pathway that is different for the 2 agents.

Such an example can be found by the use of lymphoblastoid cells from a patient with Cockayne's syndrome (*24*). Cells from this patient are hypersensitive to killing by germicidal radiation (UVC) (*25*). They are also hypersensitive to killing by radiation from an artificial fluorescent sunlamp (predominately UVB) as shown in figure 2A. However, the lymphoblastoid cell line from the patient with Cockayne's syndrome has a normal response to treatment with 8-MOP plus UVA (fig. 2B). Even though the precise defect in such cells responsible for the hypersensitivity to UVB and UVC is unknown, it is clear that this defect does not induce hyper-

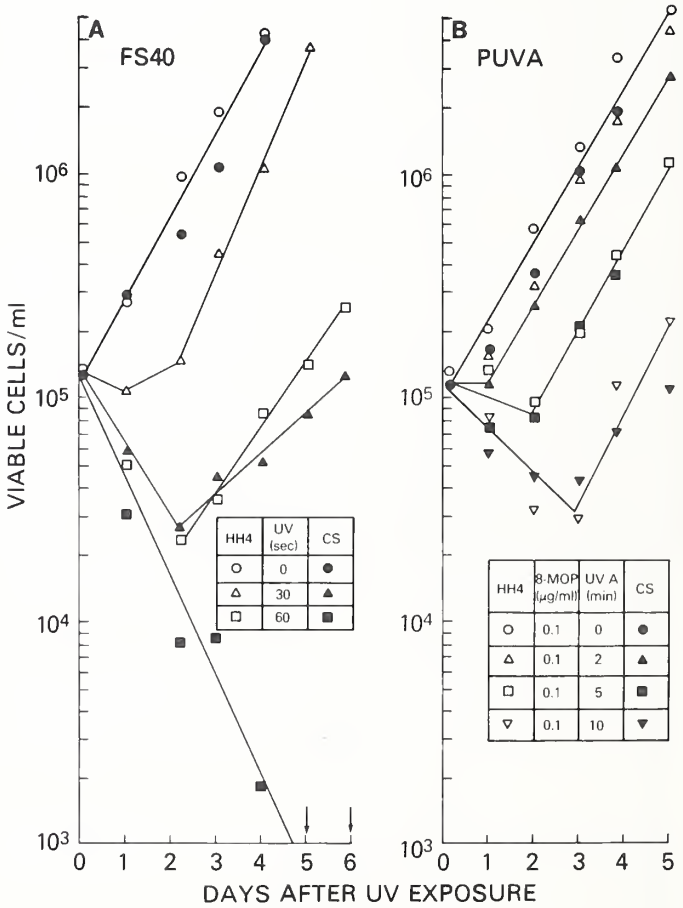

FIGURE 2.—Survival of Cockayne syndrome (CS) and normal lymphoblastoid cell lines after treatment with FS40 fluorescent sunlamp or 8-MOP plus UVA. The untreated Cockayne syndrome lymphoblastoid cell line (GM 2964) and the normal line (HH4) exhibit exponential growth in suspension culture as assessed by daily hemocytometer counts. A) Growth curves after treatment with sunlamp (UVB). B) Growth curves after treatment with 8-MOP plus UVA. Data are from (*25*) and reproduced with notification to the publisher.

sensitivity to PUVA. Thus with at least one mechanism of cell survival after PUVA that is different from a mechanism of survival after UVB, one could propose that combined UVB and PUVA treatment may be more effective than either treatment alone. Such combinations may be amenable to evaluation in vitro prior to clinical testing.

REFERENCES

(*1*) Parrish JA, Fitzpatrick TB, Tanenbaum L, et al: Photochemotherapy of psoriasis with oral methoxsalen and long-wave ultraviolet light. N Engl J Med 291:1207–1211, 1974

(*2*) Melski JW, Tanenbaum L, Parrish JA, et al: Oral methoxsalen photochemotherapy for the treatment of psoriasis: A cooperative clinical trial. J Invest Dermatol 68:328–335, 1977

(*3*) Gilchrest BA, Parrish JA, Tanenbaum L, et al: Oral methoxsalen photochemotherapy of mycosis fungoides. Cancer 38:683–689, 1976

(*4*) Roenigk HH Jr: Photochemotherapy for mycosis fungoides. Arch Dermatol 113:1047–1051, 1977

(*5*) Wade OL, Bishop JM: Cardiac Output and Regional Blood Flow. Oxford: Blackwell, 1962, pp 92–93

(*6*) Everett MA, Yeargers E, Sayre R, et al: Penetration of the epidermis by ultraviolet rays. Photochem Photobiol 5:533–542, 1966

(*7*) Parrish JA, Anderson RR, Urbach F, et al: UV-A: Biological Effects of Ultraviolet Radiation With Emphasis on Human Responses to Longwave Ultraviolet. New York: Plenum Press, 1978

(*8*) Kaidbey KH, Agin PP, Sayre RM, et al: Photoprotection by melanin: Comparison of Black and Caucasian skin. J Am Acad Dermatol 1:249–260, 1979

(*9*) Kraemer KH, Weinstein GD: Decreased thymidine incorporation in circulating leukocytes after treatment of psoriasis with psoralen and long-wave ultraviolet light. J Invest Dermatol 69:211–214, 1977

(*10*) Friedmann PS, Rogers S: Photochemotherapy of psoriasis: DNA damage in blood lymphocytes. J Invest Dermatol 74:440–443, 1980

(*11*) Strauss GH, Bridges GA, Greaves M, et al: Inhibition of delayed hypersensitivity reaction in skin (DNCB test) by 8-methoxypsoralen photochemotherapy. Lancet 2:556–559, 1980

(*12*) Lischka G, Bohnert E, Bachtold G, et al: Effects of 8-methoxypsoralen and UV-A on human lymphocytes. Arch Dermatol Res 259:293–298, 1977

(*13*) Ortonne JP, Claudy AL, Alario A, et al: Decreased circulating E rosette forming cells in psoralen UV-A treated patients. Arch Dermatol Res 258:305–306, 1977

(*14*) Haftek M, Glinski W, Jablonska S, et al: T lymphocyte E rosette function during photochemotherapy (PUVA) of psoriasis. J Invest Dermatol 72:214–218, 1979

(*15*) Dahl KB, Nyfors A, Brodthagen H: Decrease in neutrophils observed in vivo in psoriatics after PUVA therapy. Arch Dermatol Res 262:131–134, 1978

(*16*) Morison WL: In vivo effects of psoralens plus longwave ultraviolet radiation on immunity. Natl Cancer Inst Monogr 66:243–246, 1984

(*17*) Kraemer KH, Waters HL, Ellingson OL, et al: Psoralen plus ultraviolet radiation-induced inhibition of DNA synthesis and viability in human lymphoid cells in vitro. Photochem Photobiol 30:263–270, 1979

(*18*) Kraemer KH, Waters HL, Cohen LF, et al: Effects of 8-methoxypsoralen and ultraviolet radiation on human lymphoid cells in vitro. J Invest Dermatol 76:80–87, 1981

(*19*) Kraemer KH, Waters HL, Buchanan JK: Survival of human lymphoblastoid cells after DNA damage measured by growth in microtiter wells. Mutat Res 72:285–294, 1980

(*20*) Cohen LF, Kraemer KH, Waters HL, et al: DNA cross-linking and cell survival in human lymphoid cells treated with 8-methoxypsoralen and long wavelength ultraviolet radiation. Mutat Res 80:347–356, 1981

(*21*) Kraemer KH: In vitro assay of the effects of psoralens plus ultraviolet radiation on human lymphoid cells. JNCI 69:219–227, 1982

(*22*) Kohn KW, Ewig RA, Erickson LC, et al: Measurements of strand breaks and cross-links in DNA by alkaline elution. *In* Handbook of DNA Repair Techniques (Friedberg E, Hanawalt P, eds). New York: Marcel Dekker, 1981, pp 379–401

(*23*) Kraemer KH, Levis WR, Cason JC, et al: Inhibition of mixed leukocyte culture reaction by 8-methoxypsoralen and long wavelength ultraviolet radiation. J Invest Dermatol 77:235–239, 1981

(*24*) Kennedy RM, Rowe VD, Kepes JJ: Cockayne syndrome: An atypical case. Neurology 30:1268–1272, 1980

(*25*) Kraemer KH: Use of human lymphoblastoid cell lines to determine cellular hypersensitivity to physical and chemical agents. *In* The Use of Human Cells for the Assessment of Risk From Physical and Chemical Agents (Castellani A, ed). New York: Plenum Press, 1983, pp 275–283

SESSION VI

Psoralens and Ocular Cytotoxicity

Psoralens and Ocular Effects in Man and Animals: In Vivo Monitoring of Human Ocular and Cutaneous Manifestations[1, 2]

Sidney Lerman[3]

ABSTRACT—**Optical spectroscopy (fluorescence, phosphorescence, and electron spin resonance) has demonstrated the presence of 8-methoxypsoralen (8-MOP) and its potential for photobinding (to DNA and proteins) in human and animal ocular lenses. Similar photobinding in other ocular tissues, particularly the retina, has also been shown to occur in the young eye (lens has not yet developed as an effective UVA filter) and in aphakic and pseudophakic eyes. Skin biopsies were performed on a series of patients before, during, and following psoralen photochemotherapy (PUVA). High-resolution phosphorescence spectroscopy of PUVA patients enabled us to monitor the presence of 8-MOP in the skin and blood samples. These data indicated that significantly higher levels of 8-MOP accumulate within the psoriatic skin compared with uninvolved areas and demonstrated the feasibility of monitoring 8-MOP metabolism by this method. A second series of patients who had been on chronic PUVA therapy for many years were also evaluated by this technique and all patients' eyes were monitored by UV slit-lamp densitography with respect to determining lenticular photodamage in "unprotected eyes" compared with protected and untreated controls. The latter data have delineated at least 3 cases of presumptive PUVA cataracts. Phosphorescence assays on lens matter derived from individuals with a clinical diagnosis of PUVA cataracts demonstrated a photoproduct identical with the previously reported triplet noted in the lenses from experimental PUVA-treated animals. — Natl Cancer Inst Monogr 66: 227–233, 1984.**

OCULAR MANIFESTATIONS

The normal human lens is a precisely formed structure containing about 65% water and 35% organic matter made up chiefly of structural proteins. Inasmuch as the structural proteins (alpha, beta, and gamma crystallin and the insoluble protein fraction) constitute most of the dry weight of the lens, they must be vital in the transmission, absorption, and reflection of light through this organ. In the infant, the lens does not contain any chromophores capable of absorbing a significant amount of visible radiation (400–750 nm), and the potential for absorbing UV radiation longer than 295 nm resides mainly in the TRP residues present in the lens proteins and the small amount of free TRP within the lens. Aside from the minute amount of cytochromes in the lens epithelial cells, no other chromophores in the young lens are capable of absorbing radiation longer than 295 nm. The normal human cornea and aqueous humor transmit almost all of the ambient UV radiation longer than 300 nm, although there is a small but progressive increase in the percent of the longer wavelength UV radiation absorbed by the aging cornea that may be due to an accumulation of UV-induced chromophores (*1*). Thus the human ocular lens is constantly exposed to ambient near UV radiation (300–400 nm) throughout life.

Changes induced by UV in human and animal ocular tissues can be attributed to two mechanisms: a direct or intrinsic process in which the radiation is initially absorbed by specific, naturally occurring chromophores within these tissues (e.g., the nucleic acids or aromatic amino acid residues) and an indirect or photosensitized process in which the radiation is absorbed by a photosensitizing drug or other extraneous compounds.

During the past decade, a considerable amount of evidence has accumulated implicating UV radiation (between 300 and 400 nm) as an important factor in the in vitro generation of fluorescent compounds and in protein cross-linking associated with lens aging and cataractogenesis in human, mouse, and rat lenses (*1–25*). One of the consequences of cumulative photochemical damage is an increasing absorption of UV radiation and some visible light due to the presence of a series of photochemically generated lens chromophores (*1, 12, 19*). With tryptophan acting as the initial absorbing chromophore, the first photochemically generated pigment has a 340- to 360-nm excitation and 440-nm fluorescence maximum and can now serve as a photosensitizer itself, leading to a series of progressively longer wavelength (absorbing and emitting) chromophores. As the fluorescent compounds increase in concentration in the aging lens, the lens nucleus becomes yellower, and the transmission of visible as well as UV light progressively decreases with age. In about 10% of our population, this process progresses at a more rapid pace, resulting in the development of the brown (nuclear) cataract. This type of discoloration, in moderation, is actually beneficial because it enables the lens to become an effective filter for UV and short wavelength visible radiation (by the second to third decade) and thus protects the older and metabolically less efficient retina from cumulative photochemical damage which could occur during our lifetime. Laboratory studies have shown that 5 mW/cm^2 of UV radiation (longer than 325 nm) can cause irreversible retinal photodamage in the aphakic

ABBREVIATIONS: TRP = tryptophan; mW = milliwatt(s); UVA = UV radiation at 320–400 nm; UVB = UV radiation at 290–320 nm; 8-MOP = 8-methoxypsoralen.

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monkey (*26, 27*). Note that these radiation levels approach the amount of UV radiation in sunlight, which in our region (Georgia) have been measured at 2–5 mW/cm^2. These data are of particular importance for pseudophakes and older aphakic patients because current artificial intraocular lenses are excellent transmitters of UVB as well as UVA (*1*).

Aside from the effects of chronic exposure to ambient UV radiation, acute exposure to higher radiation levels can produce cortical opacities in human, rat, and rabbit lenses in vitro and in vivo (*28–32*). Thus more intense UV radiation (300 nm and longer) can induce lens changes involving the cortex, whereas chronic exposure results in changes affecting the lens nucleus. These effects appear to be dose and time related.

In addition to the demonstrated direct photochemical action of UV radiation on the ocular lens, there is the possibility of photobiologic damage by means of photosensitized reactions due to the accumulation of certain drugs within this organ (*33–46*). After the 13-mm stage of development, the ocular lens is completely encapsulated and never sheds its cells throughout life. Therefore, photobound compounds will be retained and accumulate with repeated exposure resulting in enhanced lens fluorescence and even cataract formation (*38–41*).

We have demonstrated 8-MOP in human lenses 12 hours after the ingestion of a single therapeutic dose (*41*). It can also be detected within 2 hours in rat and dogfish lenses following a 1-mg ip injection, and it diffuses out of these lenses within 24 hours if they are kept in the dark (*37, 38, 44, 45*). In the presence of photic stimuli, such as ambient room light and direct 360-nm UV irradiation, lenticular fluorescence and phosphorescence (*38, 41*) are enhanced. At 360 nm, photoaddition products can be generated with TRP as well as with lens proteins in vitro in the presence of 8-MOP and oxygen (*32, 41–43*). Such a reaction in vivo would result in the permanent retention of the photoproduct within the ocular lens. One such photoproduct has recently been demonstrated in vivo in lenses derived from rats given 8-MOP and exposed to UVA (*32, 40*). The photosensitizing action of the psoralen compounds has been correlated with the generation of cyclobutane photoaddition products with thymine in the DNA molecule (*47, 48*). A similar mechanism has been proposed for 8-MOP-photosensitized lens damage (*49*). Also, 8-MOP exerts a photodynamic action in the lens by forming photoproducts with aromatic amino acids, particularly TRP residues. Nuclear magnetic resonance analyses of the photoreaction between 8-MOP and TRP demonstrate the loss of hydrogen at the 3-, 4-, and 4′,5′-positions of 8-MOP and at the indole C_2-position of TRP, in addition to concomitant changes in the aliphatic regions of the spectra (*42, 43*). Because the positions involved in this reaction are the same as those reported in the cyclobutane photoadduct formation with thymine, the photoproduct formed between 8-MOP and TRP could involve a cyclobutane type of reaction.

We have also demonstrated that 8-MOP is capable of entering the corneal epithelium, the ciliary processes, the ocular lens, and the retina (of young rat and dogfish eyes) within 2 hours after a single ip dose of 1 mg 8-MOP (*44*). When the animals are maintained in the dark, no free 8-MOP can be demonstrated either by spectroscopic methods, autoradiography, or scintillation counting after 24 hours. However, when the animals are exposed to relatively low levels of UV radiation at approximately 365 nm, a photoreaction (or photoreactions) occurs (occur) resulting in the binding of 8-MOP within these tissues. In the ocular lens, at least 1 photoreaction product is formed between 8-MOP and TRP residues of the lens proteins as well as between 8-MOP and thymine in the nucleic acid moieties (*41–44, 49*). In the cornea, bound 8-MOP accumulates mainly in the epithelium, but because of its excellent reparative mechanisms and rapid cellular turnover (~3 days), the bound 8-MOP disappears within 3 or 4 days despite continuous UV exposure. Therefore, 8-MOP therapy should not prove to be a significant hazard to the cornea. As the mature ocular lens is a highly effective filter for UVA in most mammals including man, no photobinding of 8-MOP occurs in the mature retina. However, UVA can penetrate to the retina in aphakic and pseudophakic patients and in the eyes of young patients [in whom the ocular lens still permits significant penetration of UVA (*1, 32*)]. Photobinding of 8-MOP can also occur in these retinas (*44*). Thus it would appear that photobound 8-MOP can accumulate in the retinas and ocular lenses in animals and in patients whose lenses are not an effective UVA filter or in aphakes or pseudophakes. Repeat PUVA therapy can result in an increasing accumulation of photoproducts in these 2 ocular tissues (retina and lens). Such photoproducts would then act as new photosensitizing agents within these tissues, thereby enhancing photochemical damage when exposed to UV radiation.

Several investigators (*33–36, 40*) have documented PUVA therapy and cataract formation in experimental animals; presumptive human cataracts have also been reported (*50, 51*). We have been monitoring 3 PUVA patients with lens opacities and abnormal fluorescence levels by UV slit-lamp densitography (*52, 53*). Their opacities have not progressed after they were fitted with proper UV-filtering spectacles. Thus we could only classify them as presumptive PUVA cataracts. However, we have recently received material from 2 patients who had been on chronic PUVA therapy and whose cataracts were removed by phacoemulsification. The entire lens matter that was aspirated was frozen and sent to our laboratory for evaluation by techniques we developed to demonstrate the 8-MOP lens protein photoproduct in experimental lenses (*32, 41–44*). Spectra obtained on these human lens protein samples are shown in figures 1A and 1B demonstrating the characteristic TRP phosphorescence (as previously reported for whole lenses or lens protein solution) with a lifetime of 6.0 ± 0.2 seconds. A second phosphorescence peak with excitation at 325 nm and a lifetime of 2.0 ± 0.1 seconds is also present. It is identical with the 8-MOP lens protein photoproduct we previously demonstrated in whole lenses and protein extracts derived from rats (fig. 2) that were given 1 dose of 8-MOP; their lenses were extracted 2 hours later and exposed to UVA irradiation (<1 mW/cm^2) for 4 hours (*32, 44*). The third nonspecific phosphorescence area at 360-nm excitation is characteristic of all adult human lenses and can be attributed to some of the fluorescent chromophores that develop in this organ (*19, 24, 32*). Identical spectra were obtained on the lens matter derived from both patients. The 8-MOP photo-

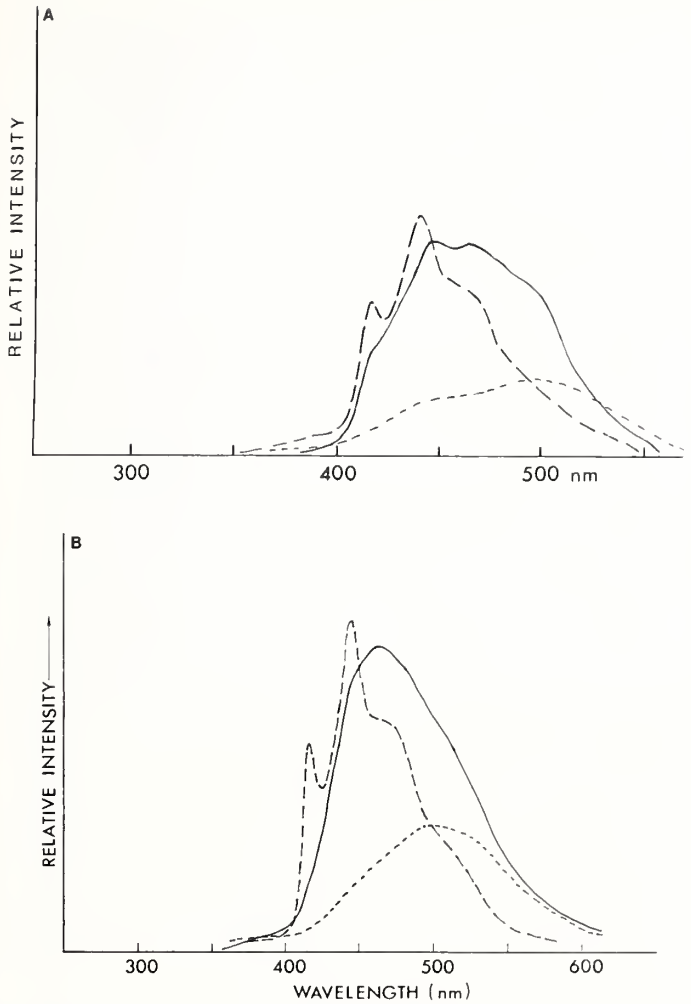

FIGURE 1.—A) Phosphorescence spectra of lens protein derived from a PUVA cataract showing characteristic TRP phosphorescence (290-nm excitation: $\tau = 6.0 \pm 0.2$ sec, ---------); 8-MOP lens protein photoproduct (325-nm excitation: $\tau = 2.0 \pm 0.1$ sec, ———); and nonspecific phosphorescence characteristic of all adult human lenses (.....) associated with some of the fluorescent chromophores (360-nm excitation). B) Phosphorescence spectra of lens protein derived from the second PUVA cataract showing similar TRP, 8-MOP photoproduct, and nonspecific phosphorescence.

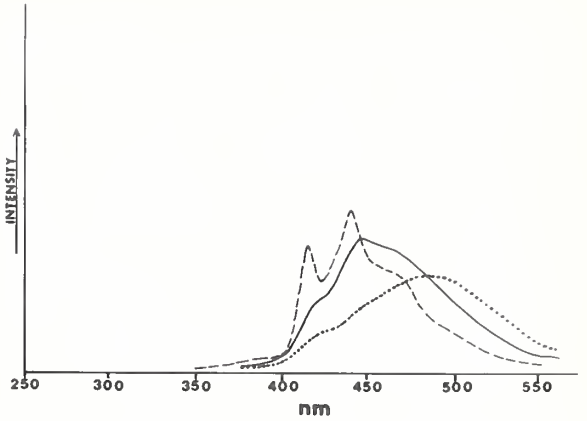

FIGURE 2.—Phosphorescence spectra of whole lens derived from rat given 1 mg 8-MOP and exposed to UVA for 24 hr showing identical 8-MOP photoproduct.

product must be associated with the lens proteins rather than DNA. The original material we obtained did not include the lens capsules and associated epithelium, and the small amount of nucleic acids from the cortical fibers in the germinative zone that might still be present would be greatly diluted by the total protein concentration. Although DNA does have a low but demonstrable phosphorescence, we could not demonstrate it in our samples even at the highest instrument gain.

Since results of laboratory studies first demonstrated enhanced fluorescence in the ocular lens associated with aging and PUVA therapy and presumptive PUVA cataracts have been reported, a method to monitor lens fluorescence in vivo has been developed (*52, 53*). A new slit-lamp densitographic apparatus (based on the Scheimpflug principle) capable of accurately and reproducibly recording visible changes in lens density as it ages has been introduced (*54*). We modified this apparatus to use UV radiation (300–390 nm) to measure and quantitate the age-related fluorescence levels in the normal lens in vivo and correlate them with our in vitro data (*1, 12, 14, 19*). Aside from demonstrating the normal age-related increase in lens fluorescence, we can also detect abnormally enhanced fluorescence caused by occupational (or accidental) exposure to above ambient levels of UV radiation. Enhanced fluorescence or abnormal fluorescence emission, or both, can also occur in patients on PUVA therapy. A clinician's failure to protect such patients properly from all UV radiation for at least 24 hours following ingestion of the drug can even result in cataract formation, as shown in figure 3. This 52-year-old patient with psoriasis was on 4 years of intermittent PUVA treatment without proper eye protection. We have now seen 3 such cataracts resulting after PUVA therapy, and several others have been reported in the literature (*50*). Although our Dermatology Clinic now provides all patients to whom PUVA is administered with proper UV-absorbing or reflecting spectacles, we have data on a series of patients who were treated before 1977, when we first demonstrated the potential for photosensitized lens damage from psoralen therapy. Their densitograms demonstrate a significant elevation of 1 of the lens fluorescence peaks (*55*).

CUTANEOUS MANIFESTATIONS

We recently developed a method whereby we can follow the accumulation and binding of psoralens within the skin. Biopsies (2–3 mm), obtained from normal untreated controls and from patients before and 2 hours after they in-

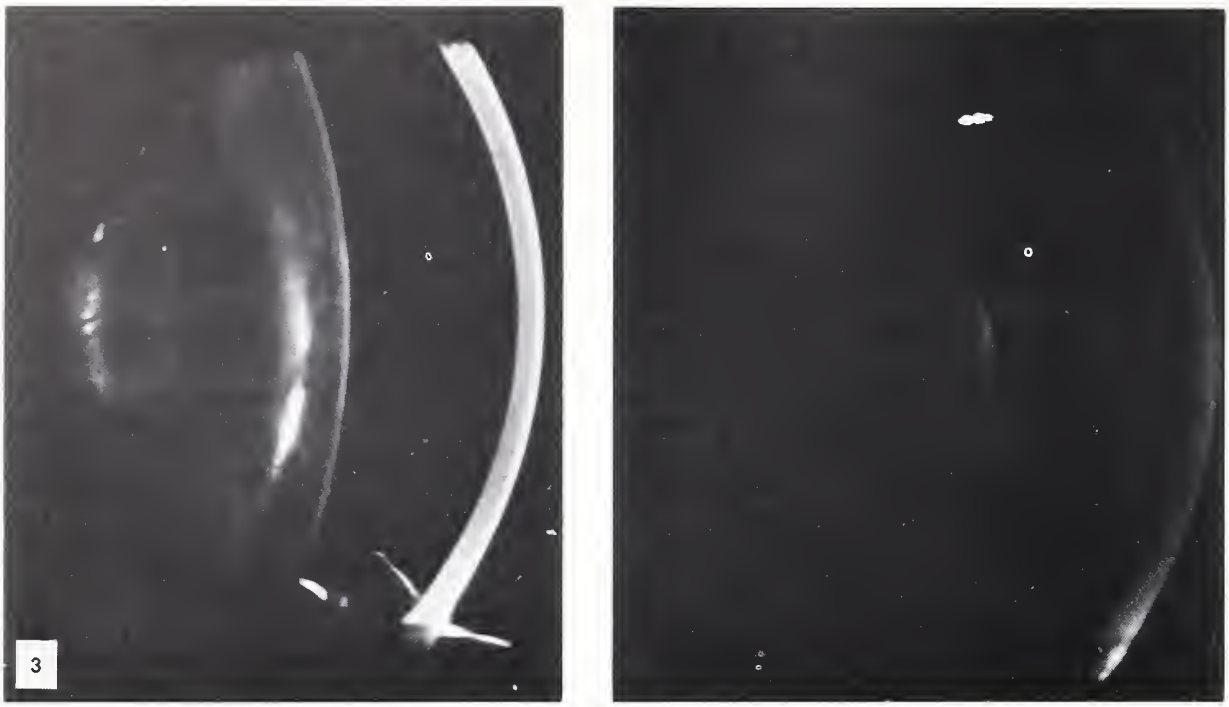

FIGURE 3.—Visible (*left*) and UV (*right*) slit-lamp photography of a 52-yr-old patient who received PUVA therapy and developed cataracts.

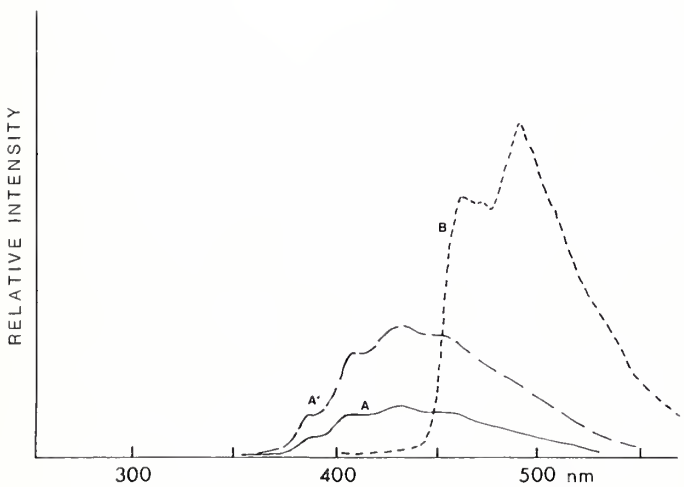

FIGURE 4.—Phosphorescence spectra of a mixture of 6.6-mg calf thymus DNA and 3×10^{-4} *M* 8-MOP in ethylene glycol showing DNA phosphorescence (A = 270-nm excitation, A′ = 302-nm excitation) and 8-MOP triplet (B) at 320-nm excitation. Same gain used for A, A′, and B.

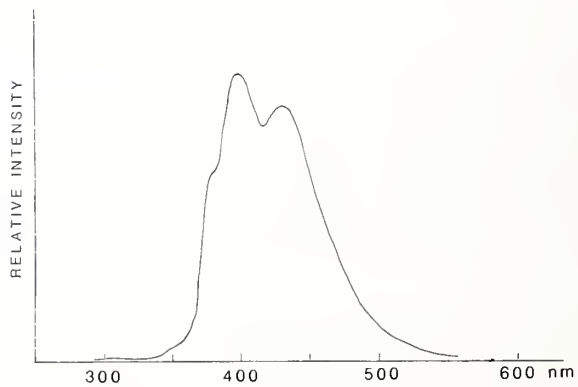

FIGURE 5.—Phosphorescence spectrum of a 2-mm biopsy derived from normal buttock skin showing mainly thymine phosphorescence (270-nm excitation).

gested 8-MOP and 1–2 hours after UVA exposure, were subjected to high-resolution phosphorescence spectroscopy (*32, 37, 42*).

Phosphorescence spectra of a mixture of DNA and 8-MOP are shown in figure 4. Normal skin samples showed phosphorescence which can be attributed mainly to the

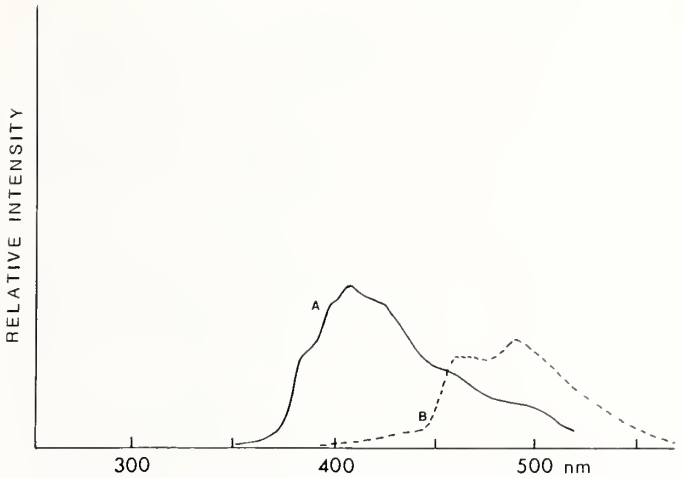

FIGURE 6.—Phosphorescence spectra of a biopsy from a psoriatic area 2 hr after 8-MOP showing the nucleic acid (A = 270-nm excitation) and 8-MOP (B = 320-nm excitation). Same gain as in figure 3.

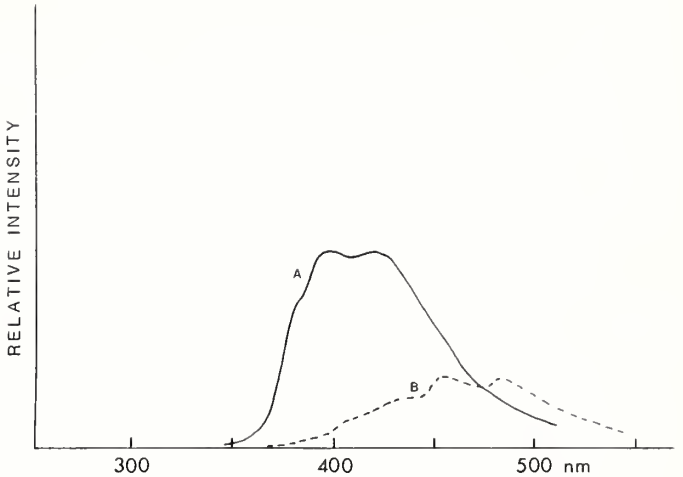

FIGURE 8.—Phosphorescence spectra of psoriatic specimen 2 hr after UVA exposure from same patient as in figures 5 and 6. *Note* 8-MOP (B) can still be detected, but intensity is significantly diminished compared with figure 6. Same gain as in figures 3 and 5.

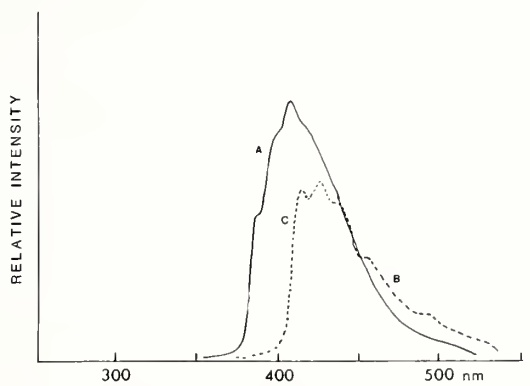

FIGURE 7.—Phosphorescence spectra of biopsy specimen from uninvolved skin 2 hr after 8-MOP ingestion from same patient as in figure 5 showing nucleic acid (A), trace of 8-MOP (B), and TRP (C). Gain was ten times that seen in figure 5.

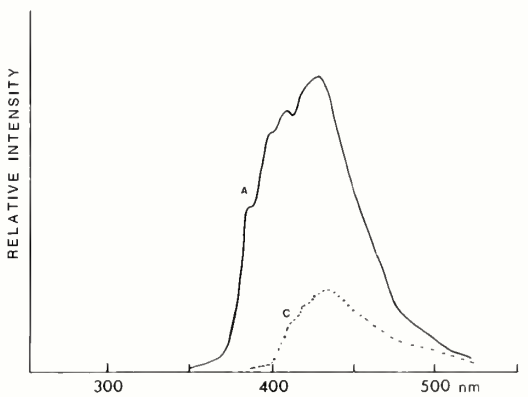

FIGURE 9.—Phosphorescence spectra of uninvolved skin area from same patient as above 2 hr after UVA exposure. The 8-MOP can no longer be detected even at maximum gain (ten times that seen in fig. 3). A is nucleic acid and C represents TRP.

thymine moiety of DNA (fig. 5). However, skin biopsies derived from patients 2 hours after they were given a therapeutic dose of 8-MOP demonstrate the 8-MOP triplet and the nucleic acid phosphorescence. We observed that 8-MOP is always present in significantly higher concentration as evidenced by peak intensity in biopsies derived from involved skin psoriatic lesions compared with uninvolved skin samples obtained on the same patient (figs. 6–9). These are preliminary data, but they indicate that we will be able to follow the fate of 8-MOP in the skin in vivo and monitor its accumulation and perhaps UVA photobinding, thereby providing spectroscopic correlation with in vitro data. This study should also help delineate the specific action of 8-MOP in human tissue, which has heretofore not been accomplished.

REFERENCES

(*1*) LERMAN S (ed): Susceptibility of ocular tissues to radiant energy. Biologic and chemical effects of ultraviolet radiation. *In* Radiant Energy and the Eye. New York: Macmillan, 1980, pp 29–186

(*2*) LERMAN S, TAN AT, LOUIS D, et al: Anomalous absorptivity of lens proteins due to a fluorogen. Ophthalmic Res 1:338–343, 1970

(*3*) ZIGMAN S: Eye lens color formation and function. Science 171:805–809, 1971

(*4*) LERMAN S: Lens proteins and fluorescence. Isr J Med Sci 8:1583–1589, 1972

(*5*) PIRIE A: Effect of sunlight on proteins of the lens. *In* Contemporary Ophthalmology (Bellows J, ed). Baltimore: Williams & Wilkins, 1972, pp 484–501

(6) SATOH K, BANDO M, NAKAJIMA A: Fluorescence in human lens. Exp Eye Res 16:167–172, 1973
(7) AUGUSTEYN RC: Human lens albuminoid. Jpn J Ophthalmol 18:127–134, 1974
(8) DILLEY KJ, PIRIE A: Changes to the proteins of the human lens nucleus in cataract. Exp Eye Res 19:59–72, 1974
(9) AUGUSTEYN RC: Distribution of fluorescence in the human cataractous lens. Ophthalmic Res 7:217–224, 1975
(10) SPECTOR A. ROY D, STAUFFER J: Isolation and characterization of an age-dependent polypeptide from human lens with non-tryptophan fluorescence. Exp Eye Res 21:9–24, 1975
(11) BANDO M, NAKAJIMA A, SATOH K: Coloration of human lens protein. Exp Eye Res 20:489–492, 1975
(12) LERMAN S: Lens fluorescence in aging and cataract formation. Doc Ophthalmol 8:241–260, 1976
(13) LERMAN S, KUCK JF, BORKMAN RF, et al: Induction, acceleration and prevention (in vitro) of an aging parameter in the ocular lens. Ophthalmic Res 8:213–226, 1976
(14) LERMAN S, BORKMAN RF: Spectroscopic evaluation and classification of the normal aging and cataractous lens. Ophthalmic Res 8:335–353, 1976
(15) KURZEL RB, WOLBARSHT ML, YAMANASHI BS: UV radiation effects on the human eye. Photochem Photobiol 26:133–167, 1977
(16) BORKMAN RF, DALRYMPLE A, LERMAN S: Ultraviolet action spectrum for fluorogen production in the ocular lens. Photochem Photobiol 26:129–132, 1977
(17) BORKMAN RF: Ultraviolet action spectrum for tryptophan destruction in aqueous solution. Photochem Photobiol 26:163–166, 1977
(18) BORKMAN RF, LERMAN S: Evidence for a free radical mechanism in aging and UV irradiated ocular lenses. Exp Eye Res 25:303–309, 1977
(19) LERMAN S, BORKMAN RF: Photochemistry and lens aging. *In* Interdisciplinary Topics in Gerontology: Gerontological Aspects of Eye Research (von Hahn HP, ed). Basel: S Karger, 1978, pp 154–182
(20) LERMAN S, YAMANASHI B, PALMER RA, et al: Photoacoustic, fluorescence and light transmission spectra of normal, aging and cataractous lenses. Ophthalmic Res 10:168–176, 1978
(21) ZIGMAN S, DATILER M, TOROZYNSHI E: Sunlight and human cataract. Invest Ophthalmol Vis Sci 18:462–467, 1970
(22) CASTINEIRAS SG, DILLON J, SPECTOR A: Effects of reduction on absorption and fluorescence of human lens proteins. Exp Eye Res 29:573–575, 1979
(23) YU NT, KUCK JF, ASKREN CC: Red fluorescence in older and brunescent human lenses. Invest Ophthalmol Vis Sci 18: 1278–1280, 1979
(24) LERMAN S: Lens transparency and aging. *In* Aging of the Lens (Regnault F, Hockwin O, Courtour Y, eds). New York, London: Elsevier/North Holland Biomedical Press, 1980, pp 263–279
(25) GARNER MH, SPECTOR A: Selective oxidation of cysteine and methionine in normal and senile cataractous lenses. Proc Natl Acad Sci USA 77:1274–1277, 1980
(26) HAM WT, MULLER HA, RUFFOLO JJ: Retinal effects of blue light exposure. *In* Ocular Effects of Non-Ionizing Radiation. Bellingham, Washington: Society of Photo-Optical Instrument Engineers, 1980, pp 46–50
(27) HAM WT, MULLER HA, RUFFOLO JJ, et al: Retinal sensitivity of the rhesus monkey to near UV radiation. Presented at the Ninth Annual Meeting of the American Society for Photobiology, Williamsburg, Va., June 14–18, 1981
(28) ZIGMAN S, VAUGHN T: Near UV light effects on the lenses and retinas of mice. Invest Ophthalmol 13:462–465, 1974
(29) PITTS DG, HACKER PD, PARR WH: Ocular Ultraviolet Effects From 295 nm to 400 nm in the Rabbit Eye. DHEW (NIOSH) Publ No. 77–175. Washington, D.C.: U.S. Govt Print Off, 1977
(30) LERMAN S: Human UV radiation cataracts. Ophthalmic Res 12:303–314, 1980
(31) THOMAS DM, SCHEPLER KL: Raman spectra of normal and ultraviolet-induced cataractous rabbit lens. Invest Ophthalmol Vis Sci 19:904–912, 1980
(32) LERMAN S, GARDNER K, MEGAW J, et al: The prevention of direct and photosensitized UV radiation damage to the ocular lens. Ophthalmic Res 13:284–292, 1981
(33) CLOUD TM, HAKIM R, GRIFFIN AC: Photosensitization of the eye with methoxsalen. Arch Ophthalmol 64:346–461, 1960
(34) _____: Photosensitization of the eye and methoxsalen. II. Chronic effects. Arch Ophthalmol 66:689–694, 1961
(35) FREEMAN RG, TROLL D: Photosensitization of the eye by 8-methoxypsoralen. J Invest Dermatol 53:449, 1969
(36) EGYED MN, SINGER L, EILAT A, et al: Eye lesions in ducklings fed ammi majus seeds. Zentralbl Vet Med Assoc 22:764, 1975
(37) LERMAN S, BORKMAN RF: A method for detecting 8-methoxypsoralen in the ocular lens. Science 197:1287–1288, 1977
(38) LERMAN S, JOCOY M, BORKMAN RF: Photosensitization of the lens by 8-methoxypsoralen. Invest Ophthalmol Vis Sci 16:1065–1068, 1977
(39) WULF HC, HART J: Accumulation of 8-methoxypsoralen in the rat retina. Acta Ophthalmol 56:284–290, 1978
(40) KOCH HR, BEITZEN R, DREMER F, et al: Effect of 8-methoxypsoralen and long UV on the rat lens. Invest Ophthalmol Vis Sci 18(Suppl):218, 1979
(41) LERMAN S, MEGAW J, WILLIS I: Potential ocular complications of PUVA therapy and their prevention. J Invest Dermatol 74:197–199, 1980
(42) _____: The photoreaction of 8-MOP with tryptophan and lens proteins. Photochem Photobiol 31:235–242, 1980
(43) MEGAW J, LEE J, LERMAN S: NMR analyses of tryptophan-8-methoxypsoralen photoreaction products. Photochem Photobiol 32:265–269, 1980
(44) LERMAN S, MEGAW J, GARDNER K, et al: Localization of 8-methoxypsoralen in ocular tissues. Ophthalmic Res 13: 106–116, 1981
(45) WULF HC, ANDREASEN MP: Concentration of ^{3}H-8-methoxypsoralen and its metabolites in the rat lens and eye after a single oral administration. Invest Ophthalmol Vis Sci 22:32–36, 1982
(46) LERMAN S, MEGAW J, GARDNER K: Allopurinol therapy and human cataractogenesis. Am J Ophthalmol 94:141–146, 1982
(47) DALL'ACQUA S, MARCIANI M, ZAMBON F, et al: Kinetic analysis of the reaction (365 nm) between psoralen and DNA. Photochem Photobiol 24:489–495, 1979
(48) SONG P-S, TAPLEY KJ JR: Photochemistry and photobiology of psoralens. Photochem Photobiol 29:1177–1197, 1979
(49) JOSE JG, YIELDING KL: Photosensitive cataractogens, chlorpromazine and methoxypsoralen, cause DNA repair synthesis in lens epithelial cells. Invest Ophthalmol Vis Sci 17:687–691, 1978
(50) CRYLIN MN, PEDVIS-LEFTICK A, SUGAR J: Cataract formation in association with ultraviolet photosensitivity. Ann Ophthalmol 12:786-790, 1980
(51) LERMAN S: Ocular phototoxicity and psoralen plus ultraviolet radiation (320–400 nm) therapy: An experimental and clinical evaluation. JNCI 69:287–302, 1982

(*52*) LERMAN S, HOCKWIN O, DRAGOMIRESCU V: In vivo lens fluorescence photography. Ophthalmic Res 13:224-228, 1981

(*53*) LERMAN S, HOCKWIN O: UV-visible slit lamp densitography of the human eye. Exp Eye Res 33:587–596, 1981

(*54*) DRAGOMIRESCU V, HOCKWIN O, KOCH HR, et al: Development of new equipment for rotating slit image photography according to Scheimpflug's principle. *In* Interdisciplinary Topics in Gerontology (Hockwin O, ed), vol 13. Basel: S Karger, 1978, pp 118–130

(*55*) LERMAN S: UV slit lamp densitography: An additional tool for prospective studies of changes in lens transparency. *In* Ageing of the Lens (Hockwin O, ed). Munich: Integra, 1982, pp 139–154

Psoralens and Ocular Effects in Humans[1]

William B. Glew[2] and Thomas P. Nigra[3]

ABSTRACT—The safety of psoralen photochemotherapy (PUVA) in relation to the eye has been an area of research at the Washington Hospital Center for the last 6 years. Our studies indicate that, with proper shielding of the eye, safety for acute lenticular damage can be established. However, the potential for cataract formation from low-dose, long-term ambient UVA exposure is not known. The guidelines for proper shielding of the eye with PUVA therapy have been established and are enumerated in the text. Our studies on 68 patients for an average length of 4 years and 7 months while they received PUVA have established that the rate in the development of cataracts in treated patients had not increased as compared with the Framingham Eye Study. — Natl Cancer Inst Monogr 66: 235–239, 1984.

Studies at the Washington Hospital Center, Washington, D.C., over the past 6 years have clarified the margin of safety of PUVA therapy in relation to acute lenticular damage in the photosensitized eye. A bioassay was developed for the measurement of nanogram quantities of photoactive unmetabolized psoralen (*1*). It was then demonstrated in vitro that, with photosensitizer levels and UVA dosages comparable to those found in the human lens, acute cellular damage could be produced (*2*). However, in acute experiments in vivo, much higher dosages are required to produce a cataract.

Natural photoprotective mechanisms are present that effectively preclude observable acute lenticular damage in vivo. The most important of these endogenous mechanisms appears to be the nonconvalent binding of the psoralen photosensitizer to human serum albumin. This reversible binding inactivates 93% of the psoralen, dramatically reducing the number of photoactively available molecules (*3*).

Because threshold dosage combinations of light and drug are reciprocally related, the less the photoactive drug level, the greater the UVA energy required to produce cellular damage. In the human eye where lens levels of 50 ng/g lens tissue are theoretically possible, UVA energy levels of at least 100 joules/cm^2 would be required to produce observable acute damage because of photosensitizer inactivation by protein binding. This is a quantity of energy considerably higher than the highest single dose used in PUVA therapy.

Other photoprotective mechanisms include the natural anatomical protection of the lens: shielding of the eye by the orbital rim and eyebrow, squinting of the lids in the sunlight, and constriction of the pupil. The cornea is transparent to UVA, transmitting about 55% in humans and so provides little shielding of the lens.

Experimental studies show that although labeled psoralen metabolites persist for more than 24 hours in the retina and lens, the photoactively available unmetabolized psoralen is diffused from the eye within 24 hours (*4*). Reversible serum protein binding in conjunction with rapid metabolism and excretion appears to be most important in this rapid reduction of photosensitivity in the eye tissue.

However, drug levels even as low as 15 ng/g lens tissue that have been measured in the human lens 3 hours after drug ingestion could, in combination with ambient UVA photons, result in incremental cellular changes. No one knows whether cellular repair mechanisms are adequate to prevent the accumulation of cytologic and enzymatic injuries, which could ultimately lead to cataract formation.

Cataracts associated with PUVA therapy for vitiligo were observed (*5*) in a 42-year-old woman who had multiple treatments with inadequate ocular protection. Dr. Sidney Lerman has data on 3 examples of lens opacities in patients inadequately shielded during PUVA therapy.

Because of this potential for cataract formation in the unshielded eye, compliance by patients treated with PUVA in the wearing of UVA opaque goggles in the PUVA chamber after ingestion of the drug and for the rest of the treatment day should be the minimum protection required. However, compliance has been a problem with patients who receive PUVA. Some do not wear the glasses during the 2 hours after drug ingestion and before irradiation in the PUVA chamber. Others object to wearing conspicuous dark wraparound glasses following PUVA chamber treatment. During the hours before and after therapy, the unshielded human lens can therefore be exposed to ambient UVA in sunlight which penetrates through window glass and the cornea.

Compliance has been improved by the recent marketing of 2 clear lenses, which are UVA opaque: the Spectra-Shield coated glass lenses and the UV 400 plastic lenses (Orcolite). These lenses, which look normal with only a slight tint and transmit most of the visible spectrum while filtering out the UVA, can be used indoors in place of dark wraparound glasses. A lighter tint wraparound has also been marketed recently.

Guidelines for shielding of eyes have been compiled and recently published as follows (*6*). They are reproduced here with permission of the publisher.[4]

ABBREVIATIONS: PUVA = psoralen plus UV radiation at 320–400 nm; 8-MOP = 8-methoxypsoralen.

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Shielding of eyes. UVA opaque goggles must be worn during treatment in the irradiation chamber. A reliable radiometer should be used to verify that no UVA is transmitted through the goggles.

During day 1 of PUVA treatment, UVA-blocking plastic wraparound glasses should be worn while outdoors from time of ingestion of the drug until bedtime. While indoors or in dim light, either the plastic wraparound glasses or clear UVA-blocking glasses should be worn. However, patients should not attempt to drive in dim light while using dark glasses.

During day 2, either the plastic wraparounds or the clear UVA-blocking glasses should be worn the entire day.

Shielding the eyes during day 1 is an absolute requirement, and on day 2 it should be encouraged but is a relative requirement.

Patients need not purchase the expensive clear glasses for indoors during day 1 or day 2; their cosmetic acceptance is superior, but all of the preceding requirements can be met by relatively inexpensive clear plastic wraparound glasses (Blak-Ray).

The following are examples of UVA-blocking spectacles for use outside of the irradiation chamber.

Wraparound glasses

Noir (Recreational Innovations Co., P.O. Box 159, South Lyon, MI 48178/313-769-5565)
Model 101 or 501 (amber sunglasses) can be worn over prescription frames
Model 102 or 502 (gray-green sunglasses) can be worn over prescription frames
Cost: $12 plus $1.35 shipping and handling

Blak-Ray (Ultraviolet Products Inc., San Gabriel, CA 91778/213-285-3123)
Model Contrast Control Spectacles UVC-303 (yellow tinted clear) can be worn over prescription frames.
Cost: $6 each

Clear UVA-blocking glasses

UV 400 (Orcolite Dioptics, Ltd., 190 West Yale Loop, Irvine, CA 92714. For ordering by local optical lab, telephone 800-423-8561 or within California call collect 213-969-1744, or 714-559-4748 for technical information.)
Model (yellow tinted)
Cost: $58-$100 plus frame for pair of finished single-vision lenses. Delivery time approximately 3 weeks. Nonprescription lenses are available at lower cost.

Human II Lens (Spectra-Shield, Inc., 327 West Arden Ave., Glendale, CA 91203/213-246-0535)
Model (coating materials applied to prescription lenses)
Cost: $35 plus postage per pair; delivery time approximately 3 weeks

Blak-Ray (clear, but wraparound, *see above* for address)

These are the recommendations for 1981 and are subject to revision and inclusion of newer technology.

A considerable task of education lies ahead for PUVA therapy teams, if they are to get 100% of the patients to shield their eyes adequately during the first 24 hours as well as during irradiation in the PUVA chamber.

Although our studies are indicative of a fairly wide margin of safety with regard to acute lenticular damage, such as that shown in the rabbit, repeated incremental subclinical cellular damage remains the major concern at this point. Recall that long-term steroid therapy for arthritis and collagen vascular disease had been in use for at least 10 years before the recognition by physicians that cataracts were a frequent side effect of this important therapy.

The reports of cataractogenesis associated with PUVA therapy in a few inadequately shielded patients and the experimentally demonstrable lenticular cytotoxicity of UVA irradiation in the presence of relatively high levels of psoralen mandate a careful clinical follow-up of patients. We conducted a study with a group of 68 patients followed over a course of PUVA therapy averaging 4 years and 7 months.

Nine of these 68 either developed cataracts or showed an increase in cataracts already present at the start of therapy. An analysis of the rate of development of cataracts in PUVA-treated patients grouped according to age and a comparison with the prevalence of cataracts in similar age groups in the Framingham Eye Study suggest that the rate of development of cataracts in this group of patients has not exceeded that which would be expected from age alone.

In the Framingham Eye Study, the prevalence of cataracts reducing vision to 20/30 or worse in the age group 52–64 years was 4.6%; 65–74, 18%; and 75–85, 46%. From this increasing prevalence in the 3 age groups, a rough estimate of the rate of development of cataracts in a normal aging population would be 1% per year in the sixth decade to 4% per year in the seventh decade.

In the eyes of our patients, we detected cataracts in 10 eyes at the start of therapy. Of these 10 (average age of patients was 53.4 yr), 3 showed no increase in cataracts, 7 showed some increase but with vision remaining 20/25 or better, and only 1 developed a cataract that reduced vision to 20/40. Of the 63 patients whose eyes were free of cataracts (average age, 45.5 yr), only 1 eye in a 65-year-old developed a cataract and reduced vision to 20/30. Thus only 2 of 68 patients developed cataracts which reduced vision to 20/30 or less, a rate of development of 3% for the 4-year, 7-month period, or 0.65% per year. If only those patients 50 years of age or over are considered, 1 of 21 patients in the 50- to 60-year group developed a cataract in 1 eye, with vision reduced to 20/40 in that eye, and 1 of 12 patients 60–70 years old developed a cataract with vision reduced to 20/30 in 1 eye. Over the period of our observations, the yearly rate of development of cataracts in these 2 age groups of patients averaged 1% and 1.7% for patients in the sixth and seventh decades, respectively. Therefore, these rates of development of cataracts in the PUVA-treated patients do not exceed those due to aging, as estimated for the age-related increase in cataract prevalence in the Framingham Eye Study.

Changes in lenticular yellowing and the rate of development of myopia in an older group of patients treated with PUVA for nuclear sclerosis was also studied because of the probable relationship of sunlight exposure to the formation of pigmented nuclear cataracts (*7, 8*). The eyes of 27 patients with nuclear sclerosis at the start of therapy, whose average age was 55 years, only 4 eyes showed a change of refraction of −.25 or −.50 sphere, which is an insignificant amount and

rate of change. No increase in lenticular yellowing was detected on biomicroscopy. In addition, no nuclear sclerosis was seen to develop in 82 eyes with clear lenses at the start of therapy. The lack of significant changes observed for up to 6 years suggests that the eyes of patients treated with PUVA who follow the guidelines for photoprotection are not at increased risk for the development of nuclear sclerotic cataracts.

Also apparently significant is that, in the group of 126 eyes with no cataracts at the start of the study, a cataract developed in only 1 eye of a 65-year-old patient. Thus in the group under 60 years of age, cataracts developed only in those eyes which already had incipient cataract formation before PUVA therapy was initiated.

Experimental studies demonstrate that acute lenticular damage can be produced in vivo if dosages of UVA irradiation and psoralen photosensitizer are sufficiently high. These threshold values for acute in vivo cytotoxicity are considerably higher than those ordinarily used in PUVA therapy. A few reports of cataract formation have been associated with PUVA therapy in patients whose eyes were inadequately shielded. Therefore, we believe the cumulative effects of repeated increments of photosensitization or, alternatively, increased tissue susceptibility to photosensitization in certain individuals is related to cataract formation. These cumulative effects and increased tissue susceptibility underscore the importance of adequate UVA photoprotection for patients receiving PUVA.

Compliance with the guidelines for UVA photoprotection has been good in a carefully followed group of 68 patients who received PUVA for an average of 4 years and 7 months. In this group, the incidence of cataract formation was minimal; however, the rate of cataract development appears to be no greater than that associated with age alone.

REFERENCES

(*1*) GLEW WB, ROBERTS WP, MALININ GI, et al: Quantitative determination by bioassay of photoactive 8-methoxypsoralen in serum. J Invest Dermatol 75:230–234, 1980

(*2*) GLEW WB: Determination of 8-methoxypsoralen in serum, aqueous, and lens: Relation to long-wave ultraviolet phototoxicity in experimental and clinical photochemotherapy. Trans Am Ophthalmol Soc 77:464–514, 1979

(*3*) GLEW WB, MCKEEVER G, ROBERTS WP, et al: Photochemotherapy and the eye: Photoprotective factors. Trans Am Ophthalmol Soc 78:243–254, 1980

(*4*) MALININ GI, GLEW WB, ROBERTS WP, et al: Kinetics of (14C-5) 8-methoxypsoralen uptake by UVA-irradiated and nonirradiated rabbit eye tissues. Res Commun Chem Pathol Pharmacol 32:223–234, 1981

(*5*) CYRLIN MN, PEDVIS-LEFTICK A, SUGAR J: Cataract formation in association with ultraviolet photosensitivity. Ann Ophthalmol 12:786–90, 1980

(*6*) FARBER E, EPSTEIN J, NALL L, et al: Current status of oral PUVA therapy for psoriasis: Eye protection revisions. J Am Acad Dermatol 6:851–855, 1982

(*7*) ZIGMAN S, DATILES M, TOREZYNSKI E: Sunlight and human cataracts. Invest Ophthalmol Vis Sci 18:462–467, 1979

(*8*) HILLER R, GIACOMETTI L, YUEN K: Sunlight and cataract: An epidemiologic investigation. Am J Epidemiol 105:450–459, 1977

DISCUSSION

J. E. Hearst: Dr. Lerman's and Dr. Glew's papers on the ocular effects of psoralens are now open for discussion. I wonder if we can start the discussion by asking Dr. Stern and maybe Dr. Wolff or Dr. Christophers to make a less than 1-minute conclusive statement about their experiences with eye studies and their patient groups.

R. Stern: In about 1,100 patients treated with PUVA, who had examinations at least 3 years apart, we had 35 reported cataracts. Twenty of those 35 patients had at least 1 eye with vision below 20/40. We compared data from the Framingham Eye Study with ours and, at our age distribution, could find no evidence for an increase in cataract formation or a decrease in best-corrected visual acuity, nor did we find a dose–response relationship with respect to those patients who developed cataracts as opposed to those who did not.

E. Christophers: We have no evidence for cataract development in our patients treated with PUVA except that related to age.

Hearst: Does anyone have questions for Dr. Lerman or Dr. Glew?

T. B. Fitzpatrick: Dr. Lerman, what evidence do you have that PUVA is the cause of cataracts?

S. Lerman: If you take lenses, i.e., cataractic lenses, from patients with treatment other than PUVA, you do not see the photoproduct.

Fitzpatrick: That is right.

Lerman: The 2 lenses we obtained have the photoproduct that can be shown in the laboratory. I emphasize again, a good deal of confusion is apparent. I am not talking about acute cataracts, which require high doses at high exposure levels. When you are looking at an acute cataract, you are looking at an end result. I am speaking of phototoxicity with low levels of psoralen bound to the lens protein. You now have a lens which has 1 more photosensitizer in it; the lens itself develops a series of intrinsic photosensitizers with age. That is how we develop longer wavelength-absorbing chromophores.

When psoralen is added, you have another new extrinsic photosensitizer, which can enhance photodamage, and certain patients will develop cataracts. Fortunately, most patients, if adequately protected, will not develop them because photobinding of the 8-MOP is prevented by UV-filtering glasses, and the unbound 8-MOP is readily diffused from the lens. Furthermore, it does not enter the lens bound to protein because it would not penetrate the aqueous humor. In the aqueous humor, 8-MOP is free and not bound to protein to any significant extent. If photobound to protein or DNA, it cannot leave the lens. The lens retains all of its cells after the capsule is formed at the 13-mm stage. Therefore, anything that enters and is bound can act on a cumulative basis over a long period. Damage is not acute but requires years to become manifest.

The same process can occur in the retina. You can see photic stimulation to the aphakic or pseudophakic retina or in the youngster whose lens is still a good transmitter of UVA. Thus photoproducts can form in the retina and remain there. As you know, the retina does not regenerate. I am convinced that a potential hazard exists, but it can be

avoided if you provide patients with proper UV-absorbing or reflecting glasses. I do not like the dark glasses because patients refuse to wear them at home. Even fluorescent indoor lighting is sufficient to cause photobinding.

With regard to myopia, which someone mentioned, I would not expect such changes because myopia does not necessarily occur concomitantly with lens discoloration. It is a separate process, and the discoloration may or may not occur with acute myopic change due to so-called nuclear sclerosis.

W. L. Morison: I have questions for Dr. Glew. Are there any clinical features of human cataracts that occur from PUVA which may make them readily detectable? Do you expect to find any particular features of this kind? That is the first question. Secondly, how do you measure phototoxicity in the lens?

W. B. Glew: I believe that we do not know all the possible kinds of changes that might occur on lens tissue due to the recombination of 8-MOP and UVA. However, I do recall that, with Dr. Lerman's patients, the effect was greater in the anterior than in the posterior cortex. The acute effect that we produced in our experimental animals was anterior subcapsular. In fact, it was a proliferation of the lens epithelium, as we demonstrated on electron microscopy. Little (punctate) white opacities, which others have also reported, were proliferations of the lens epithelium posteriorly into the cortex.

If we see a rash of PUVA-related cataracts 10 years from now, I think they will probably be difficult to distinguish from those resulting from aging. I will let Dr. Lerman speculate about that. Dr. Stern should continue close observation of his 1,100 patients for as long as possible because the numbers of cataracts in this group will be increased. Careful analysis of occurrence will enable his cooperative groups to make intelligent conclusions about whether the incidence is greater with the PUVA group than with a comparable group in the population.

In answer to your speculation about the drug leaving the lens, I think our work shows that a differential in the affinity of the drug is likely, an affinity of the molecules for the serum albumin molecule as opposed to lens protein. The aqueous humor has a small amount of protein. The drug probably diffuses and enters and exits different compartments. When we measured the lethal photosensitization in *Staphylococcus* and used aqueous humor instead of a balanced salt solution, the lethal effect was reduced by about one-half. We assume it was due to the binding of psoralen by the small amount of protein; not much protein is needed to produce that effect.

Lerman: I think the time has come to disprove the misconception that one can simply look at a cataract and by its appearance determine whether it was caused by PUVA treatment, ionizing radiation, or some other agent. One cannot determine this by looking at a lens with a slit lamp. That will only tell you about the presence of an opacity and its location. The lens constantly grows throughout our lives. The opacity caused by the insult will be located in different portions of the lens depending on the age at which the insult occurs. In younger people, you expect the lens opacity caused by a specific insult (let us say PUVA) to be in a certain location and that it will move inward and perhaps posteriorly with time. For a clinician to expect to look at lenses with the conventional slit lamp and determine the pathogenesis is usually a complete waste of time.

Presently, the only way you can avoid that mistake is to measure fluorescence in vivo and show abnormal fluorescence peaks, which can be correlated experimentally in vivo and in vitro with photobound 8-MOP. This can be done, and if we would screen all the patients, we would have sufficient data on those who are protected. Nothing happens when patients are protected. If you see abnormal or enhanced fluorescence in any, I predict many of those patients will enventually develop cataracts unless they wear UV-protective glasses. Cataract development is a slow, cumulative process which requires years. Obviously, if you expose an animal to a large amount of UV, you will not need psoralen; you will give it a cataract anyway.

G. Lazarus: Could you tell me what happens between the deposition of the psoralen and the UVA and the development of the cataract?

Lerman: Psoralen is photobound to the nucleic acid and the lens protein. With prolonged or continuous treatment a new photochemical hazard is introduced that will accelerate cataractogenesis.

Lazarus: What is cataractogenesis?

Lerman: Cataractogenesis is a process by which any agent (drug, radiation, etc.) causes an opacity that is visible and interferes with vision.

Lazarus: Is this a cross-linking phenomenon or an alteration in the collagen fibrils?

Lerman: Collagen is only in the capsule. The lens is composed of soluble proteins and an insoluble protein fraction, i.e., a mass of protein. Photobinding can cause cross-linking of the soluble protein chains to form more insoluble protein and also generate the fluorescent chromophores.

H. Wulf: From 1957 to 1962, 60 patients were treated with psoralen for vitiligo. They did not protect themselves with sunglasses or any other type of shield, and they were asked to go out in the sun. We recently reexamined the eyes of 40 of these patients and found no pathologic conditions.

R. Brickl: I would like to ask whether anyone has an idea what the action spectrum for possible side effects in the eye might be. The reason why I ask relates to patient compliance. Whether the patient will wear those glasses for 24 hours or so will depend mainly on the kind of glasses recommended. Is it sufficient to exclude UV at 380 nm or below? Exclusion should be possible with practically colorless glasses or plastic material. I have tested some of them. Probably, they would be worn more consistently than dark-colored glasses, which patients will not use because visibility during work is reduced.

Lerman: At Emory University Woodruff Medical Center, we recommend either the Blak-Ray, which lets all visible light through; the Silor, Univisor UV 400, which lets most of the visible light through; or the Spectra-Shield coating, if the patient has glass lenses and has complied. We discovered that when you give patients the dark-colored lenses the compliance decreases, so we do not use them. These are the best types (in the form of a goggle) to recommend to the patients. When they are worn for the first 24 hours, we have no problem with compliance.

You made a statement that you have 60 patients who did

not develop cataracts, which indicates that there are biologic variations among people. Everyone does not have to develop a disease before we should be concerned about a potential hazard. We have evidence that this disease is a possibility; photodamage to the lenses is enhanced. The hazard that is present can easily be prevented, but I cannot understand what kind of evidence retrospective or prospective studies would provide. Of course, every patient does not develop cataracts, nor does every diabetic develop cataracts. The fact that we have hundreds of diabetics who fail to develop them does not prove that diabetes and cataract formation are unrelated.

SESSION VII

Photoimmunologic Aspects of Psoralen Photochemotherapy

In Vivo Effects of Psoralens Plus Longwave Ultraviolet Radiation on Immunity[1, 2, 3]

Warwick L. Morison[4]

ABSTRACT—Treatment with 8-methoxypsoralen plus UV radiation at 320–400 nm (PUVA) alters immune function in experimental animals and humans. In guinea pigs, the treatment can suppress the development of delayed hypersensitivity to an injected antigen and the development of contact hypersensitivity locally at the site of exposure to radiation and systemically at a nonexposed site. The mechanisms of these changes are unknown but may involve certain alterations that occur after treatment in Langerhans cells. In normal human subjects, PUVA treatment can alter the distribution of circulating T- and B-lymphocytes, and this effect appears to be dose related. The distribution and function of T-lymphocytes in patients with psoriasis are also altered by PUVA treatment. These effects are greater with high, cumulative exposure doses of the treatment. Suppression of contact hypersensitivity also has been observed in patients with mycosis fungoides and psoriasis treated with PUVA. Autoimmune phenomena do not appear to be a side effect of the treatment. Although immunosuppression may be a mechanism of action by which PUVA treatment exerts its therapeutic effect on disease, it can also result in adverse effects after long-term treatment. — Natl Cancer Inst Monogr 66: 243–246, 1984.

Treatment with 8-MOP and subsequent exposure to UVA can alter immune function in experimental animals, both locally at the site of exposure of the skin to radiation and systemically at distant unexposed sites. The immune system is well represented in the skin: Langerhans cells in the epidermis belong to the monocyte–macrophage lineage and are involved in antigen uptake, processing, and presentation; lymphocytes are present in the dermis; and the mast cells of the dermis are involved in immediate hypersensitivity and possibly other immune responses. In addition to these fixed elements, lymphocytes, eosinophils, polymorphonuclear leukocytes, and antibodies percolate through the lymphatics and blood vessels of the skin. Psoralens appear to enter all cells, and UVA penetrates the epidermis and reaches the dermis. Therefore, all the various elements of the immune system in the skin are, for short or long periods, in a position to be exposed directly to PUVA. Apart from a direct effect, exposure to it may influence immune function at distant sites by way of photoproducts and mediators released from the treated skin.

Most of the studies of the effects of PUVA on immune function in vivo have been descriptive; few have dealt with the mechanism of action of the treatment. Furthermore, many studies have been conducted on patients with various diseases, which themselves may affect immune function. These two shortcomings prevent accurate interpretation of the significance and implications of the observations that have been made; much work needs to be done to further our understanding of the mechanisms of PUVA treatment.

STUDIES IN ANIMALS

Treatment with PUVA produces suppression of several types of immune responses in animals. In the guinea pig, erythemogenic doses of PUVA suppressed delayed hypersensitivity to an injected antigen (*1*). Both local and systemic suppression of this response was observed, but the suppression was greater at irradiated than at nonirradiated sites. Treatment with PUVA before immunization was more effective in suppressing delayed hypersensitivity than that given between the time of immunization and challenge. This observation suggests that the treatment mainly affects the induction phase of the immune response. Suberythemogenic doses of PUVA administered to guinea pigs also produced systemic suppression of delayed hypersensitivity to an injected antigen (*2*). Associated with this suppression is the finding that lymphocytes from the treated animals were less responsive in vitro to the antigen and a mitogen than lymphocytes from untreated animals. Interestingly, treatment with 8-MOP alone produced suppression of the in vivo responses to the antigen but did not alter the in vitro responses.

Contact hypersensitivity to DNCB in the guinea pig is also suppressed by PUVA treatment. This effect was observed when the sites of induction and elicitation were within the area exposed to radiation (*3*), when only the site of elicitation was exposed (*4*), and when induction and elicitation were performed at distant nonirradiated sites (*5*). The animals received multiple PUVA treatments, commencing at the time of sensitization, that resulted in a moderate to

ABBREVIATIONS: 8-MOP = 8-methoxypsoralen; UVA = UV radiation at 320–400 nm; PUVA = 8-MOP plus UVA; DNCB = dinitrochlorobenzene; UVB = UV radiation at 290–320 nm; SLE = systemic lupus erythematosus; ANA = antinuclear antibodies.

[1] Presented at the Conference on Photobiologic, Toxicologic, and Pharmacologic Aspects of Psoralens held in Research Triangle Park, North Carolina, March 1–3, 1982.

[2] Supported by the National Cancer Institute, Department of Health and Human Services, under contract No. N01-C075380 with Litton Bionetics, Inc. The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the United States Government.

[3] Animals were maintained under the guidelines set forth by the National Institutes of Health Policy on Humane Care and Use of Animals and by the Animal Welfare Act in facilities accredited by the American Association for Accreditation of Laboratory Animal Care.

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marked erythemal response. Because of the design of the studies, it is not clear whether the effect of PUVA was exerted on the induction or elicitation phase of the immune response, although a local effect on elicitation was suggested by 1 study (*3*). However, PUVA treatment, even in highly erythemogenic doses, did not suppress elicitation of contact hypersensitivity in animals with established sensitivity to DNCB (*3*). Erythemogenic doses of PUVA also prolonged the survival time of full-thickness allografts in rabbits (*6*) when the donor and recipient sites for the graft were exposed (*1*). The survival time of the grafts was increased by slightly more than 50%, which is less than that observed with many other immunosuppressive agents.

The mechanism of suppression of these cell-mediated immune responses is unknown. One possibility is a local effect of PUVA on Langerhans cells that results in defective processing and presentation of antigens and a preferential induction of suppressor cell pathways. Such a mechanism has been suggested as the explanation for UVB-induced suppression of contact hypersensitivity in the mouse. Fewer than normal Langerhans cells, as identified by the adenosine triphosphatase reaction, were found at the site of exposure to radiation in guinea pigs treated with PUVA (*7*). In addition, a dose of PUVA that produced suppression of contact hypersensitivity in the guinea pig (*3*) sharply reduced the number of Langerhans cells identified on 1-μm histologic sections (unpublished observations). However, the number of Langerhans cells at distant nonirradiated sites was not altered. Thus an alteration in the number or function, or both, of Langerhans cells may produce the local effects of PUVA treatment, but another mechanism probably is responsible for the systemic effects.

STUDIES IN NORMAL HUMANS

Studies on the effect of PUVA treatment on immune function in normal human subjects have been confined to examination of the distribution and function of peripheral blood lymphocytes. A single whole-body treatment with PUVA (*8*) that resulted in a marked erythemal response was associated with a decrease in the proportions of circulating T-cells (identified by E-rosette formation) and B-cells (identified by surface-membrane immunoglobulin). The absolute number of circulating lymphocytes was unaffected, and thus there was a corresponding increase in null cells in the circulation. These changes were detected 30 minutes after exposure, were maximal at 12–16 hours, and returned to pretreatment levels by 72 hours after irradiation. Doses of PUVA that resulted in little or no erythema had similar but less noticeable effects on circulating lymphocytes. The functional activity of lymphocytes, as judged by their response to graded doses of phytohemagglutinin was not altered by PUVA treatment. These findings are in agreement with 2 earlier studies in which a small decrease in the proportion of circulating T-lymphocytes was observed after exposure to PUVA once (*9*) and four times (*10*). In the latter study, the proportion of T-lymphocytes returned to pretreatment levels after 8 exposures to the treatment. Investigators who conducted these studies appear to have used doses of PUVA below the threshold for erythema.

The mechanism of these alterations is unknown. However, the rapid reversibility of the changes suggest that PUVA treatment may produce an alteration in the function of the surface membrane of the cells. Death of T- and B-lymphocytes or sequestration of damaged cells out of the circulation are less likely explanations.

STUDIES IN PATIENTS WITH DISEASE

The effect of PUVA therapy on immune function in patients with various diseases has attracted considerable attention. This interest developed from the observation that, during PUVA treatment of mycosis fungoides, the neoplastic lymphoid cells were eliminated from the skin. Since then, some researchers have observed that lymphocytes involved in the nonmalignant disease process of lichen planus are also eliminated from the skin by PUVA treatment. Whether these lymphoid cells are killed as a result of the treatment or redistributed to other sites in the body is not known. Also, PUVA treatment may alter immune responses in patients with other diseases for whom it is used because there is some evidence for the involvement of immune mechanisms in the pathogenesis of all diseases in which this therapy has been beneficial. Therefore, PUVA may act by way of an effect on normal or abnormal immune responses in some diseases and by a toxic effect on lymphocytes in the skin in other conditions.

The effect of PUVA on circulating lymphocytes has been examined in patients with psoriasis and other diseases. Immediately following exposure to UVA in the course of PUVA therapy, DNA synthesis was diminished in unstimulated lymphocytes obtained from patients with psoriasis (*11*). A diminished proportion of T-cells in the peripheral blood was found in patients with various diseases being treated with PUVA therapy (*12*). In contrast with that finding, a progressive increase in the proportion of these cells in the peripheral blood was observed during PUVA therapy for psoriasis (*13*). However, other investigators have failed to find any alteration of circulating T-cells during weeks (*14*) or months (*15*) of PUVA therapy. In patients with psoriasis, the proportion of circulating B-lymphocytes was unchanged by PUVA therapy (*10, 12, 14*). The response of lymphocytes following stimulation with phytohemagglutinin was unaffected (*13, 15*) or diminished (*14, 16*) at an early stage during a course of therapy. These apparent discrepancies among different studies are probably attributable to several factors. Psoriasis alone affects lymphocytes, and the number of circulating E-rosette-forming cells is diminished in patients with active disease (*9, 13*). The time of blood collection, relative to the last exposure to PUVA, varied among studies or was not specified. Finally, PUVA therapy is not standardized, and, presumably, the doses of PUVA administered to patients varied a great deal. The results of our study in normal subjects (*8*) suggest that both the time of blood collection and the dose of PUVA are important variables.

During the first few weeks of PUVA therapy, we found only minor alterations in lymphocyte function in patients with psoriasis, and these changes were transient even though treatment was continued (*14*). However, we continued monitoring the effects of PUVA therapy on lymphocyte function in patients with psoriasis to determine whether alterations appeared with progressively higher cumulative exposure doses of this treatment. Recently, 10 patients, selected because they had received more than 200 treatments

over a period of 2–6 years, were studied (*17, 18*). Cumulative exposure doses of UVA ranged from 1,700 to 6,000 joules/cm^2. Blood samples were taken 4–7 days after the last PUVA treatment. Lymphocytes from all 10 patients gave abnormally low responses following stimulation with graded doses of phytohemagglutinin, concanavalin-A, and pokeweed mitogen, compared with those obtained with lymphocytes from untreated patients and normal control subjects. A decrease in the proportion of circulating T-lymphocytes was also detected in the patients treated with PUVA and found to be attributable to a selective loss of helper-inducer cells. A correlation was observed between the results of mitogen and T-cell subset studies: A low level of helper-inducer cells was found in patients with a decrease in the response to phytohemagglutinin as well as pokeweed mitogen. The results of this study suggest that long-term PUVA therapy is associated with marked alterations in the function and distribution of circulating lymphocytes. These findings raise questions as to whether these effects are reversible once PUVA therapy is stopped and whether clinical evidence of altered in vivo immunity will be found.

A few observations have been made of the effect of PUVA therapy on contact hypersensitivity in patients with psoriasis and other diseases. Sensitization with DNCB was impaired in patients treated with PUVA compared with normal control subjects (*19, 20*). Patients with psoriasis who were untreated or treated with anthralin and UVB also had an impaired response to DNCB sensitization, although this was less marked than the impairment observed in patients treated with PUVA (*20*). Impaired elicitation of contact hypersensitivity (*21*) and delayed hypersensitivity to recall antigens (*22*) has been reported in patients with mycosis fungoides, but these observations were not made under controlled conditions. A possible mechanism for local suppression of contact hypersensitivity by PUVA has been suggested by the observation that this treatment diminishes the number of Langerhans cells at the site of exposure to UVA (*23*).

Two theoretical concerns about the potential toxicity of PUVA therapy are exacerbation of SLE when PUVA is inadvertently used in patients with subclinical or undiagnosed disease and induction of an SLE-like syndrome in patients in whom this disease would not otherwise develop. Because PUVA interacts with DNA, antigenicity of that molecule could be altered and lead to the development of antibodies to DNA. Such antibodies are found in patients with SLE, but at present what other derangements are necessary to produce clinical SLE are unknown. Detection of circulating ANA is a sensitive, but not specific, test for SLE that has been used to screen patients treated with PUVA. At 14 centers, 1,023 patients had 2 or more ANA determinations over a 2-year period. When first and last tests were compared, the incidence of positive tests for ANA was not significantly different (*24*). In other smaller studies, more positive ANA determinations were reported during the course of PUVA therapy (*25, 26*). However, it is likely that these findings merely reflect repetition of a sensitive test known to give fluctuating results within populations. Three case reports of the development of SLE or a similar syndrome have been published (*27–29*), but a cause-and-effect relationship cannot be established from these anecdotal observations. The possibility still remains that exposure to PUVA therapy could precipitate SLE in a predisposed patient, but it now appears unlikely that the treatment will produce this disease in patients who do not have a predisposition for it.

Bullous pemphigoid is another disorder with an immunologic pathogenesis that has been reported in patients treated with PUVA (*30–32*). This disorder is known to be associated with psoriasis, which was the disease being treated with PUVA, and can be triggered by exposure to UVB. Interestingly, but not surprisingly, PUVA therapy can trigger bullous pemphigoid; a predisposition to the disorder is probably present in such patients.

CONCLUSIONS

Although PUVA treatment can alter immunologic function, the scope of the changes, the mechanisms underlying them, and their significance are largely unexplored. Some areas have not been studied such as the effect of PUVA treatment on mast cell function and humoral immunity. Most observations are descriptive in nature, and their pathophysiology has not been examined. Furthermore, the relationships of these observations to the known beneficial and adverse effects of PUVA therapy require careful study.

As has already been suggested, the beneficial effect of PUVA therapy in various diseases may be mediated by alterations in immune function. Observations made so far suggest that such alterations probably involve the suppression of immune responses and that this immunosuppression may not be specific for any particular response. Thus in the course of producing suppression of an immune response that mediates the disease being treated, PUVA therapy may also suppress normal immune responses. This latter unwanted but possibly inevitable effect of the treatment may cause adverse side effects such as infections and neoplasia. Cutaneous neoplasia is the only side effect of the treatment observed so far that may be linked to nonspecific immunosuppression. However, opportunistic infections and lymphoid neoplasia may occur after long-term treatment.

The effects of PUVA therapy on immune function in humans have attracted considerable interest, but the possible effects of UVB phototherapy on immunity have been largely ignored. This deficiency needs to be corrected because the 2 treatments are interchangeable in some diseases; the risk:benefit ratios of the 2 treatments are difficult to compare in the absence of information on the effects of UVB phototherapy on immune responses in humans.

REFERENCES

(*1*) Morison WL, Parrish JA, Woehler ME, et al: Influence of PUVA and UVB radiation on delayed hypersensitivity in the guinea pig. J Invest Dermatol 76:484–488, 1981

(*2*) Vella Briffa D, Parker D, Tosca N, et al: The effect of photochemotherapy (PUVA) on cell-mediated immunity in the guinea pig. J Invest Dermatol 77:377–380, 1981

(*3*) Morison WL, Parrish JA, Woehler ME, et al: The influence of ultraviolet radiation on allergic contact dermatitis in the guinea pig. II. Psoralen/UVA radiation. Br J Dermatol 104:165–168, 1981

(*4*) Meyer CH Jr, Grundmann HP, Weiss H: Inhibitory effect of 8-methoxypsoralen plus UVA (PUVA) on the systemic induction of contact sensitivity to dinitrochloro-

benzene (DNCB) in guinea pigs. Arch Dermatol Res 269:189–195, 1980

(*5*) MORISON WL, KRIPKE ML: Systemic suppression of contact hypersensitivity by ultraviolet B radiation or methoxsalen/ultraviolet A radiation in the guinea pig. Cell Immunol 85:270–277, 1984

(*6*) MORISON WL, PARRISH JA, WOEHLER ME, et al: The influence of PUVA and UVB radiation on skin-graft survival in rabbits. J Invest Dermatol 75:331–333, 1980

(*7*) OKAMOTO H, HORIO T: The effect of 8-methoxypsoralen and long-wave ultraviolet light on Langerhans cells. J Invest Dermatol 77:345–346, 1981

(*8*) MORISON WL, PARRISH JA, BLOCH KJ, et al: In vivo effects of PUVA on lymphocyte function. Br J Dermatol 104: 405–413, 1981

(*9*) HAFTEK M, GLINSKI W, JABLONSKA S, et al: T lymphocyte E-rosette function during photochemotherapy (PUVA) of psoriasis. J Invest Dermatol 72:214–218, 1979

(*10*) CORMANE RH, HAMERLINCK F, SIDDIQUI AH: Immunologic implications of PUVA therapy in psoriasis vulgaris. Arch Dermatol Res 265:245–267, 1979

(*11*) KRAEMER KH, WEINSTEIN GD: Decreased thymidine incorporation in circulating leukocytes after treatment of psoriasis with psoralen and long-wave ultraviolet light. J Invest Dermatol 69:211–214, 1977

(*12*) ORTONNE JP, CLAUDY A, ALARIO A, et al: Impairment of thymus-derived rosette-forming cells during photochemotherapy (psoralen-UVA). Arch Dermatol Res 262:143–151, 1978

(*13*) FRAKI JE, ESKOLA J, HOPSU-HAVU VK: Effect of 8-methoxypsoralen plus UVA (PUVA) on lymphocyte transformation and T-cells in psoriatic patients. Br J Dermatol 100:543–550, 1979

(*14*) MORISON WL, PARRISH JA, BLOCH KJ, et al: Transient impairment of peripheral blood lymphocyte function during PUVA therapy. Br J Dermatol 101:391–397, 1979

(*15*) HARPER RA, TAM DW, VONDERHEID EC, et al: Normal T lymphocyte function in psoriatic patients undergoing methoxsalen photochemotherapy. J Invest Dermatol 72: 323–325, 1979

(*16*) FRIEDMANN PS, ROGERS S: Photochemotherapy of psoriasis: DNA damage in blood lymphocytes. J Invest Dermatol 74:440–443, 1980

(*17*) MORISON WL, PARRISH JA, MOSCICKI R, et al: Abnormal lymphocyte function following long-term PUVA therapy for psoriasis. J Invest Dermatol 76:303, 1981

(*18*) MOSCICKI RA, MORISON WL, PARRISH JA, et al: Reduction of the fraction of circulating helper-inducer T cells identified by monoclonal antibodies in psoriatic patients treated with long-term psoralen/ultraviolet-A radiation (PUVA). J Invest Dermatol 79:205–208, 1982

(*19*) STRAUSS GH, GREAVES M, PRICE M, et al: Inhibition of delayed hypersensitivity reaction in skin (DNCB test) by 8-methoxypsoralen photochemotherapy. Lancet 2:556–559, 1980

(*20*) MOSS C, FRIEDMANN PS, SHUSTER S: Impaired contact hypersensitivity in untreated psoriasis and the effects of photochemotherapy and dithranol/UV-B. Br J Dermatol 105:503–508, 1981

(*21*) VOLDEN G, MOLIN L, THOMSEN K: PUVA-induced suppression of contact sensitivity to mustine hydrochloride in mycosis fungoides. Br Med J 2:865–866, 1978

(*22*) KUBBA R, BAILIN PL, ROENIGK HH JR: Immunologic evaluation in mycosis fungoides. Arch Dermatol 116:178-181, 1980

(*23*) FRIEDMANN PS: Disappearance of epidermal Langerhans cells during PUVA therapy. Br J Dermatol 105:219–221, 1981

(*24*) STERN RS, MORISON WL, THIBODEAU LA, et al: Antinuclear antibodies and oral methoxsalen photochemotherapy (PUVA) for psoriasis. Arch Dermatol 11:1320–1324, 1979

(*25*) BJELLERUP M, BRUZE M, FORSGREN A, et al: Antinuclear antibodies during PUVA therapy. Acta Derm Venereol (Stockh) 59:73–75, 1978

(*26*) KUBBA R, STECK WD, CLOUGH JD: Antinuclear antibodies and PUVA photochemotherapy. Arch Dermatol 117:474–477, 1981

(*27*) DOMKE HF, LUDWIGSEN E, THORMANN J: Discoid lupus erythematosus possibly due to photochemotherapy. Arch Dermatol 115:642, 1979

(*28*) EYANSON S, GREIST MC, BRANDT KD, et al: Systemic lupus erythematosus. Arch Dermatol 115:54–56, 1979

(*29*) MILLNS JL, MCDUFFIE FC, MULLER SA, et al. Development of photosensitivity and an SLE-like syndrome in a patient with psoriasis. Arch Dermatol 114:1177–1181, 1978

(*30*) ABEL EA, BENNETT A: Bullous pemphigoid. Arch Dermatol 115:988–989, 1979

(*31*) THOMSEN K, SCHMIDT H: PUVA-induced bullous pemphigoid. Br J Dermatol 95:568–569, 1976

(*32*) ROBINSON JK, BAUGHMAN RD, PROVOST TT: Bullous pemphigoid induced by PUVA therapy. Br J Dermatol 99:709–713, 1978

Effects of Methoxsalen Plus Near-Ultraviolet Radiation or Mid-Ultraviolet Radiation on Immunologic Mechanisms[1, 2, 3]

Margaret L. Kripke[4]

ABSTRACT—Skin cancers induced in mice by UVB, i.e., 280–320 nm radiation, are highly antigenic. They grow progressively in UVB-irradiated hosts because of certain specific immunologic alterations that are induced in the mice. Comparative studies of the immunologic aspects of carcinogenesis by UVB or methoxsalen plus UVA, i.e., 320–400 nm radiation (PUVA), formed the basis for the following conclusions: 1) Skin cancers induced by PUVA in C3H/HeN mammary tumor virus-negative mice are not highly antigenic, in contrast to those induced by UVB; 2) PUVA-induced tumors also differ from those induced by UVB, in that they do not exhibit preferential growth in UVB-irradiated mice; 3) PUVA treatment of mice, unlike UVB, does not induce susceptibility to the transplantation of UVB-induced tumors; 4) both UVB and PUVA treatments suppress the induction of contact hypersensitivity by a mechanism that involves suppressor lymphocytes. — Natl Cancer Inst Monogr 66: 247–251, 1984.

During the past several years, studies in our laboratory have been directed toward understanding the role of the immune system in photocarcinogenesis. Skin cancers induced in inbred mice by UVB have provided a particularly interesting model for such investigations because they are highly antigenic in comparison to tumors induced by chemical carcinogens. In fact, many of these UVB-induced tumors are rejected immunologically upon transplantation to unirradiated syngeneic mice (*1, 2*). This finding raised the question of how such antigenic tumors could escape immunologic destruction during their induction and growth in the primary host. Work from our laboratory and other laboratories [reviewed in (*3*)] has shown that 1) UVB irradiation of mice causes systemic immunologic alterations and 2) these alterations probably are responsible for the progressive growth of the antigenic UVB-induced skin cancers.

As an extension of these studies, we have begun to investigate some of the immunologic parameters of skin carcinogenesis by PUVA in mice. Although the questions of PUVA carcinogenesis and PUVA-induced immunosuppression are important in their own right, our efforts have been directed toward comparing the effects of PUVA treatment with those established previously for UVB. The reasons behind this orientation are twofold: First, we are interested in assessing whether the findings with UVB concerning the involvement of immunologic factors in skin carcinogenesis can be generalized to include other cutaneous carcinogens. Because it induces at least some biologic responses in the skin that are similar to those resulting from exposure to UVB and because PUVA carcinogenesis and UVB carcinogenesis both involve repeated treatments over a long period, PUVA seemed like a logical choice for these studies. Furthermore, because methoxsalen is known to damage DNA directly after exposure to UVA, one can use this treatment to determine whether any of the observed immunologic effects of UVB might result from nonspecific DNA damage in a particular type of target cell. In addition, the existence of a family of psoralen compounds with different cellular and molecular targets provides a potential approach for analysis of the mechanisms underlying the immunologic alterations produced by UV radiation.

With these goals in mind, we have begun to investigate some of the antigenic properties of skin cancers induced by PUVA treatment and some of the immunologic consequences of PUVA treatment in mice. Although these studies are far from complete, they already have provided some interesting contrasts between the effects of the UVB and PUVA treatments.

INDUCTION AND ANTIGENICITY OF PSORALEN PLUS NEAR-ULTRAVIOLET RADIATION-INDUCED TUMORS

Previous studies established that skin cancers induced in $C3H^-$ mice by repeated exposure to UVB have two distinctive properties. They are highly antigenic and grow progressively in syngeneic mice that have been exposed to a short course of UVB. The high degree of antigenicity is demonstrated by transplantation of the tumors to normal and immunodeficient syngeneic mice. A high proportion of

ABBREVIATIONS: UVB = UV radiation at 280–320 nm (mid); PUVA = psoralen (methoxsalen) plus UV radiation at 320–400 nm (near); $C3H^-$ = C3H/HeN mammary tumor virus negative; kJ = kilojoule(s); CHS = contact hypersensitivity; DNFB = 1-fluoro-2,4-dinitrobenzene.

[1] Presented at the Conference on Photobiologic, Toxicologic, and Pharmacologic Aspects of Psoralens held in Research Triangle Park, North Carolina, March 1–3, 1982.

[2] Supported by the National Cancer Institute, Department of Health and Human Services, under contract No. N01-C075380 with Litton Bionetics, Inc. The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the United States Government.

[3] Animals were maintained under the guidelines set forth by the National Institutes of Health Policy on Humane Care and Use of Animals and by the Animal Welfare Act in facilities accredited by the American Association for Accreditation of Laboratory Animal Care.

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the primary UVB-induced skin cancers are rejected by the normal recipients but grow progressively in immunosuppressed mice. We (*4*) have used this type of transplantation test to assess the antigenicity of PUVA-induced skin cancers and to compare the ability of these skin cancers to induce immunologic rejection with that of UVB-induced tumors.

For these studies, skin cancers were induced by UVB or PUVA in female C3H⁻ mice. We shaved the mice with electric clippers weekly to remove their dorsal fur. The UVB-induced skin cancers were produced by exposure of the mice for 1 hour three times/week to UV radiation from unfiltered FS40 sunlamps obtained from Westinghouse Electric Corporation (Pittsburgh, Pa.). The average incident dose-rate of the radiation was approximately 1 $J \cdot m^{-2} \cdot second^{-1}$; about 80% of this energy was emitted within the wavelength range from 280 to 340 nm. The PUVA-induced skin cancers were produced in the mice by ip injections of 0.4 mg methoxsalen in 0.5 ml methylcellulose five times/week. One hour after each injection, the mice were exposed to UVA from PUVA fluorescent bulbs made by Sylvania Corporation (Millburn, N.J.) for 2, 5, or 15 minutes. The radiation was filtered through Mylar to eliminate wavelengths below 320 nm. Over the wavelength range from 320 to 400 nm, the average incident dose-rate of the radiation was approximately 10 $J \cdot m^{-2} \cdot second^{-1}$.

Of 15 skin tumors induced by PUVA treatment, 12 were examined histologically. Three of these were squamous carcinomas and 9 were fibrosarcomas. Morphologically, these tumors were indistinguishable from the skin tumors produced by chronic UVB irradiation. No skin tumors developed on mice that were treated with methoxsalen alone or UVA alone. Twelve PUVA-induced tumors and 19 UVB-induced tumors were tested for their ability to grow in normal or immunosuppressed syngeneic mice. Primary tumors were removed, cut into 1-mm^3 fragments, and 1 fragment was transplanted sc to each of 5 normal mice and 5 mice immunosuppressed by adult thymectomy plus sublethal (450 rad) whole-body X-irradiation. None of the 19 UVB-induced tumors grew in any of the normal recipients, whereas all of them grew in 1 or more of the immunosuppressed mice, as was expected from previous studies. In contrast, all of the PUVA-induced tumors grew with a high efficiency in both normal and immunosuppressed mice. Thus based on the criterion of the ability to induce immunologic rejection in unimmunized syngeneic mice, the PUVA-induced tumors are not highly antigenic. In this regard, they clearly are different from the skin cancers induced by UVB.

The PUVA-induced and UVB-induced tumors also differ in their ability to exhibit preferential growth in UVB-irradiated mice, relative to that in normal recipients. This behavior is characteristic of UVB-induced skin cancers, and it is attributable to the presence in UVB-irradiated mice of suppressor T-lymphocytes that prevent the rejection of these tumors. Thus cells of UVB-induced tumors injected at doses that are rejected by normal mice generally grow progressively in UVB-irradiated mice. With some of the PUVA-induced tumors one can find a dose of cells that will grow rapidly in immunosuppressed mice but at a much slower rate in normal animals. Even under these conditions, the PUVA-induced tumors do not exhibit enhanced growth in UVB-irradiated mice. This suggests that, if the PUVA-induced tumors have tumor-specific transplantation antigens, these antigens as a group differ in their specificity from those occurring on UVB-induced tumors.

COMPARISON OF THE IMMUNOSUPPRESSIVE EFFECTS OF MID-ULTRAVIOLET RADIATION AND PSORALEN AND NEAR-ULTRAVIOLET TREATMENTS

Because UVB irradiation of mice prevents immunologic rejection of UVB-induced tumors, we (*5*) wanted to determine whether PUVA treatment would produce the same immunosuppressive effect. Mice treated 5 days/week for 6 weeks with 0.4 mg methoxsalen ip followed 1 hour later by 9 kJ UVA/m^2 were given a sc injection of cells from a UVB-induced fibrosarcoma. At the dose of cells injected, the UVB-induced tumors grew progressively in UVB-irradiated mice and in immunodeficient mice, but they were rejected in both untreated mice and in the PUVA-treated animals. Tumor rejection also was observed in mice treated topically with 0.1 mg methoxsalen plus up to 27 kJ UVA/m^2 five times/week for 6 weeks. Both PUVA regimens were chosen because they produce an amount of gross phototoxicity equal to or greater than that produced by the UVB. These findings contrast with those reported by Roberts et al. (*6*), who concluded that PUVA treatment, like UVB treatment, converted mice to a state of susceptibility to the growth of UVB-induced tumors. However, this difference could be attributed to the fact that unfiltered UVA lamps were used in the latter study, and, therefore, the animals actually were treated with a combination of PUVA and UVB.

Our findings indicate that PUVA and UVB treatments differ in that PUVA treatment does not alter the immunologic rejection of UVB-induced tumors. This could be because PUVA treatment failed to activate the suppressor lymphocyte pathway, which is activated by UVB treatment (*3*). Alternatively, PUVA treatment could activate the suppressor lymphocyte pathway, but the antigenic specificity of these suppressor cells could differ from those produced by UVB irradiation. To distinguish between these possibilities, one must determine whether PUVA-treated mice have suppressor lymphocytes with antigenic specificity for PUVA-induced tumors. Nonetheless, these experiments demonstrate that PUVA treatment does not result in a generalized immunosuppression because the PUVA-treated mice can reject syngeneic UVB-induced skin cancers. This conclusion is supported by preliminary experiments indicating that the PUVA-treated mice also make a normal antibody response to sheep red blood cells (Jensen PJ: Unpublished data). On the other hand, the response to contact-sensitizing antigens is impaired by PUVA treatment. This is analogous to our previous finding (*7*) that UVB suppresses CHS.

EFFECTS OF PSORALEN AND NEAR-ULTRAVIOLET RADIATION ON CONTACT HYPERSENSITIVITY

A single exposure of the shaved dorsum of mice to UVB 3 or more days before an application of DNFB or 1-chloro-1,3,5-trinitrobenzene to the shaved abdomen reduces the CHS reaction to these agents. The reaction is elicited 5–7 days after sensitization by one's painting the sensitizer on

the ear surfaces and measuring the ear swelling 24 hours later. We protected the ears during the irradiation by covering them with black insulating tape. The amount of suppression observed is directly proportional to the dose of UV radiation. At the time suppression is observed in the UV-irradiated mice, antigen-specific suppressor T-lymphocytes can be found in their spleens (*8*). Correlative evidence suggests that the induction of suppressor T-lymphocytes occurs because of an alteration in the presentation of antigens by macrophages in the UV-irradiated mice (*7, 9*). The nature of this alteration in antigen-presenting cell function is unknown, however.

Our recent experiments have been designed to address the question of whether these events also occur in PUVA-treated mice. The C3H$^-$ mice were given a single PUVA treatment consisting of an ip injection of 0.4 mg methoxsalen in 0.5 ml 2% gelatin. After 30–45 minutes, the shaved dorsal skin of the mice was exposed to Mylar-filtered PUVA bulbs for 90 minutes (54 kJ UVA/m^2). The ears were shielded with tape during the UVA treatment. (The ears of the control mice were taped also.) This dose of UVA was selected because it produces approximately the same amount of gross skin damage as that observed 1–2 weeks after a single 3-hour exposure to UVB. The results of a representative experiment shown in table 1 indicate that PUVA treatment, but not UVA alone or methoxsalen alone, suppressed the CHS reaction to about the same extent as the UVB. Neither UVB nor PUVA suppressed the elicitation phase of the CHS reaction, as is shown by the finding that treatments given after sensitization do not interfere with the expression of CHS.

Spleen cells taken from UVB- or PUVA-treated mice 6 days after sensitization with DNFB were injected iv into normal animals. These recipients were unable to develop CHS to a sensitizing dose of DNFB (table 2). This suggests that mice treated with PUVA, like those exposed to UVB, produce suppressor lymphoid cells in response to contact allergens.

Several conclusions can be drawn from these studies, even though the work is incomplete: 1) Skin cancers induced in mice by PUVA do not have the same antigenic properties as those induced by UVB, even though the tumors are indistinguishable histologically. 2) The PUVA treatment has immunosuppressive effects in mice, as evidenced by suppression of the CHS reaction. 3) Only certain immune responses are suppressed by PUVA treatment, whereas other immune responses appear to be unaffected. 4) The suppression of CHS by UVB and by PUVA is accompanied by the appearance of suppressor lymphoid cells. However, additional studies are required if we are to determine whether the same cellular mechanisms are responsible for the suppression produced by these 2 treatments.

TABLE 1.—*Suppression of CHS by UVB or PUVA treatment*

Group	Treatment	DNFB	Ear swelling ±SEM, cm^{-3} [a]	Percent suppression [b]	P [c]
1	None	−	2.2±0.3	—	<0.001
2	None	+	12.3±1.0	—	
3	Methoxsalen	+	11.5±0.7	8	NS [d]
4	UVA	+	10.3±1.0	20	NS [d]
5	PUVA	+	5.6±0.8	66	<0.001
6	UVB	+	2.9±0.7	94	<0.001

[a] Values indicate ear swelling 24 hr after challenge ± SEM (10 mice/group).

[b] Percentage is (ear swelling of test group) − (ear swelling of group 1)/(ear swelling of group 2) − (ear swelling of group 1) × 100.

[c] Probability is not different from group 2 by the *t*-test.

[d] NS = not significant.

TABLE 2.—*Transfer of suppression to DNFB with spleen cells from UVB- or PUVA-treated mice*

Group	Treatment	DNFB	Ear swelling ±SEM, cm^{-3} [a]	Percent suppression [b]	P [c]
1	UVB	+	5.7±1.4	74	<0.01
2	PUVA	+	5.6±2.0	68	<0.02
3	None	+	11.9±0.9	—	
4	None	−	2.6±0.4	—	<0.001
5	Group 1 cells	+	3.0±0.7	73	<0.01
6	Group 2 cells	+	3.2±0.8	70	<0.01
7	Group 3 cells	+	6.9±0.9	18	NS [d]
8	No cells	+	8.2±1.3	—	
9	No cells	−	1.1±0.4	—	<0.001

[a] Values indicate mean ear swelling of 5 mice 24 hr after challenge ± SEM.

[b] *See* footnote *b* in table 1.

[c] Probability is not different from group 3 (groups 1, 2, and 4) or group 8 (groups 5, 6, 7, and 9) by the *t*-test.

[d] NS = not significant.

REFERENCES

(*1*) KRIPKE ML: Antigenicity of murine skin tumors induced by ultraviolet light. J Natl Cancer Inst 53:1333–1336, 1974

(*2*) _____: Latency, histology, and antigenicity of tumors induced by ultraviolet light in three inbred mouse strains. Cancer Res 37:1395–1400, 1977

(*3*) _____: Immunologic mechanisms in UV radiation. Adv Cancer Res 34:69–106, 1981

(*4*) KRIPKE ML, MORISON WL, PARRISH JA: Induction and transplantation of murine skin cancers induced by methoxsalen plus ultraviolet (320–400 nm) radiation. JNCI 68: 685–690, 1982

(*5*) _____: Differences in the immunologic reactivity of mice treated with UVB or methoxsalen plus UVA radiation. J Invest Dermatol 76:445–448, 1981

(*6*) ROBERTS LK, SCHMITT M, DAYNES RA: Tumor susceptibility generated in mice treated with subcarcinogenic doses of 8-methoxypsoralen and long-wave ultraviolet light. J Invest Dermatol 72:306–309, 1979

(*7*) NOONAN FP, KRIPKE ML, PEDERSEN GM, et al: Suppression of contact hypersensitivity in mice by ultraviolet radiation is associated with defective antigen presentation. Immunology 43:527–533, 1981

(*8*) NOONAN FP, DE FABO EC, KRIPKE ML: Suppression of contact hypersensitivity by UV radiation and its relationship to UV-induced suppression of tumor immunity. Photochem Photobiol 34:683–689, 1981

(*9*) GREENE MI, SY MS, KRIPKE ML, et al: Impairment of antigen-presenting cell function by ultraviolet radiation. Proc Natl Acad Sci USA 76:6591–6595, 1979

DISCUSSION

C. Jansen: As we heard, whether PUVA treatment will be giving our patients cancer is not only a question of how many mutations are produced. It is as much a question about to what extent the patients' defense mechanisms might be compromised.

We heard Dr. Kripke talking about specific immune defense responses, but we also have nonspecific defense mechanisms. The natural killer cell is presently considered one of the most important nonspecific antitumor mechanisms. It is sort of a first-line defense, so when mutations arise in skin cells, this defense mechanism may determine whether the mutated cells will go on producing actual tumors.

We have been studying the natural killer cell activity in patients undergoing oral PUVA treatment, with K562 cells as targets. When the natural killer cell activity was measured after each 10 PUVA sessions in a group of patients with psoriasis, a slight decrease of basal natural killer activity was seen during the first 30 treatments, but thereafter the activity reverted to normal. In parallel assays, into which 1,000 U of α-interferon was added, the resulting augmentation of natural killer cell activity increased in magnitude during the course of PUVA therapy.

Our study corroborates the data presented by Dr. Morison that showed clinical PUVA treatment indeed can influence peripheral lymphocyte functions in man. Also, we have demonstrated that, with prolonged PUVA treatment, this killer cell activity and its augmentation by interferon seem to be preserved or even augmented. This can be considered good news for PUVA, if you want to put it that way.

K. M. Halprin: I think that Dr. Morison also showed that the first treatment, 1 treatment, also did not decrease the number of killer cells or null cells.

W. L. Morison: Pamela Jensen in our laboratory has studied natural killer cell activity in the PUVA-treated patients who received more than 200 treatments, normal individuals who received a single erythemogenic dose of PUVA, and normal individuals who were given a single erythemogenic dose of UVB. We found no alteration in comparison to normal cells.

K. Wolff: I have a question for Dr. Kripke. Would you care to speculate publicly on the nature of your suppressor cells that you transferred?

M. L. Kripke: As I mentioned, in the UVB-treated animals, the suppressor cell for CHS is a T-lymphocyte which appears to be antigen specific. The experiments that we have done so far with suppressor cells from PUVA-treated animals involve nylon wool purification of the cells. Ordinarily, if you incubate mouse lymphocytes on a nylon wool column and then elute the unbound cells, this population is highly enriched for T-lymphocytes. If we use this procedure with the spleen cells from the PUVA-treated animals, we reduce activity. That result suggests that the suppressor cell in the animals treated with PUVA may either be a B-lymphocyte or a macrophage, both of which adhere to the nylon wool column. However, this is not definitive evidence because some classes of T-cells adhere to nylon wool, and also our cell populations have not been characterized fully.

Recently, we have also tried to remove the suppressive activity with monoclonal antibodies directed against T-lymphocytes and also by adherence to plastic to see whether it is due to macrophages. I cannot give you the results at this time.

T. J. Harrist: How were these tumors diagnosed? For example, how was the distinction made between spindle cell squamous carcinoma and fibrosarcoma?

Kripke: This is an argument that I avoid at all costs. I could not tell a fibroblast from a red blood cell under the microscope, and I rely heavily on people who are involved in mouse pathology. The diagnosis has been fibrosarcoma rather than spindle cell carcinoma, but I am not sure that anybody really has tried to make a distinction.

Harrist: We have also noted undifferentiated spindle cell carcinomas in mice treated with PUVA; however, I think they represent squamous cell carcinoma. We are in the process of performing transmission electron microscopy to ensure this point. Have you studied Langerhans cells in skin irradiated by PUVA or UVB for alteration in number or function?

Kripke: I believe no one in our laboratory has yet looked at Langerhans cells in PUVA-treated animals. We examined mice exposed to UVB from both FS40 sunlamps and also a monochromator to determine the effects on Langerhans cells. Dr. Frances Noonan has recently completed a study on the wavelength dependence of morphologic alterations in Langerhans cells in skin after UVB. She found the same types of morphologic alterations that have been described by Dr. Wolff's group and by Bergstresser and Streilein in Dallas, i.e., a loss of dendritic morphology of the cells and a reduction in the number of adenosine triphosphatase-positive cells.

What is interesting about our studies is that if we look at the skin on the unirradiated sites of the animals where we are actually applying the contact sensitizer, the Langerhans cells appear to be normal in appearance and number.

Harrist: Lastly, lichen planus and mycosis fungoides are responsive to PUVA. These disorders are characterized by increased numbers of Langerhans cells and infiltration by helper-inducer lymphocytes, as defined by monoclonal antibody studies. Perhaps there is a specific effect on either of the 2 cell types that lead to lesion regression.

Morison: Dr. Harrist and I have studied Langerhans cells in guinea pigs at the site of exposure and at a nonexposed site and found the same results as have been detected in the mouse, i.e., that the number of Langerhans cells has been decreased at the exposed area, but we observed no alterations in Langerhans cells at nonexposed sites.

J. H. Epstein: What is the timing of the irradiation, Dr. Kripke? When did you apply the antigen compared with when you irradiated with both PUVA and UVB?

Kripke: This is a most important question because of the dramatic time effect involved here. First, the time course is similar for a single UVB or PUVA treatment; that is one fact we know. If you irradiate and then wait 24 hours and apply the contact sensitizing agent, there is no suppression whatsoever, only a perfectly normal response. It takes at least 2 to 3 days for whatever is happening to happen in these animals. After a single treatment with either PUVA or UVB, the reaction is suppressed maximally when the sensitizer is

applied about 3 days after the irradiation. It remains low for 2 weeks after the single exposure, and it recovers to a normal level at about 3 weeks after the initial treatment.

More interesting is what happens with chronic treatment, which we have investigated with UVB but not with PUVA. With chronic UVB radiation, the animals eventually recover their ability to respond to contact sensitizing agents, even with continued exposure to UVB. This implies that either a mediator for the reaction is being depleted from the skin or that the skin has thickened to the point that whatever the photoreceptor is, it is not being exposed to radiation any longer.

D. M. Carter: My question deals with the animal models themselves and attempts to translate this information to the human situation. We have talked about both animals and humans. I am curious to know from those who work with animal models if it is true that the animals do not or cannot develop metastatic cancers produced by some other means. We should know the answer to this to interpret your data. Secondly, can one produce by any means basal cell cancers in mice of this or another strain?

Kripke: As far as I know, at least in the mouse, basal cell tumors, or what is commonly classified as a basal cell carcinoma, does not occur. The epidermal tumors are generally classified as squamous cell carcinomas in the mouse. I do not know whether rats get basal cell carcinoma or not.

Wolff: Yes, they do.

Epstein: Yes. You do that chemically in rats.

Kripke: The other question deals with metastasis. The reason I wanted to pursue that point with Dr. Forbes is that metastasis in a primary tumor system in the mouse is highly unusual. That is why I was so curious about this report that exists in the literature that PUVA induces metastatic tumors in the mouse. Apparently that is an unconfirmed observation. The tumors may well be metastatic because with the UVB-induced tumors that we studied, a tumor that has been grown in tissue culture or one that has been transplanted will metastasize perfectly well upon intravenous or even subcutaneous injection into an appropriately immunosuppressed host. They will metastasize to various body sites, including back to the skin, but for some probably interesting reason, this does not normally occur in the primary host, in the mouse. Does anybody want to comment on this?

Epstein: We have observed just a few metastases over the years. Our experience, at least with the mice we studied, has been that they are not good "metastasizers." It is true whether you use 7,12-dimethylbenz[*a*]anthracene or UV or viral stimuli; the results are the same. They just do not metastasize in the mice readily. You may have a strain that does, but ours do not.

Recommendations on Psoralen Use and Needs for the Future: A Discussion[1, 2]

M. A. Pathak: Before we submit our recommendations and suggestions to the National Toxicology Program of the National Institute for Environmental Health Sciences, I would like to express my personal appreciation to all the people who contributed to the success of this scientific meeting. Secondly, I would like to express my personal thanks to the following chairmen of the various scientific sessions: Drs. D. M. Carter, F. Dall'Acqua, J. H. Epstein, T. B. Fitzpatrick, L. I. Grossweiner, K. M. Halprin, L. C. Harber, A. Kornhauser, G. S. Lazarus, H. B. Matthews, T. P. Nigra, J. A. Parrish, K. C. Smith, and M. Wick. I also acknowledge the assistance of Elder Pharmaceuticals of Bryan, Ohio, which contributed to the scientific success of this meeting.

We would like to have each of you give your recommendations and suggestions. Also, if you would prefer not to voice your opinions, please write to us; we would be greatly obliged to include some of your recommendations.

I recommend that Dr. Kendric Smith open this session.

K. Smith: Your first mistake was to invite me to the meeting. As someone said, "I am not prepared, but it is not going to stop me." Obviously, my perspective on this problem is at the level of molecular DNA repair and mutagenesis, and I recognize first that even at the basic radiation biologic and mutagenicity levels, much is unknown at the present time. We do not understand properly how bacteria repair their DNA, and little has been done over the years on improving our understanding of how psoralens are repaired. The psoralen that has been studied mainly is TMP, and that has biologic properties and clinical effects quite different from 8-MOP. A tremendous amount of fundamental and basic research remains to be done even at the bacterial level, or perhaps especially at the bacterial level, because of the availability of bacterial mutants that are deficient in specific types of repair.

Earlier in our discussions I mentioned some preliminary data that Dr. Grossweiner and I have acquired on different mutants with 3 monoadduct-forming psoralens. They clearly seem to be repaired by different mechanisms, and that is presumably without any cross-links. People tend to think that a one-hit mutation curve is relevant to monoadducts and a two-hit curve implies cross-links. Now, that is certainly a valid first approximation, but previously I mentioned that one can also get a 2-hit mechanism of mutagenesis from UV radiation, which probably is not due to cross-links but to 2 independent hits within the same gene that is being assayed, thus giving 2-hit kinetics.

I appreciate the problems encountered clinically because you have many patients, and you must go forward and do the best you can. I do think that somewhere perhaps we have to go back and study some of these psoralen compounds at the basic level again and be careful that we are not prejudiced by current dogma. In DNA repair, we have gone through several stages of dogmatism that have blocked or "clouded men's minds." Fortunately, after 4 or 5 years, a particular dogma is disproved, and then some progress is made for a while until the next dogma appears and crystallizes our thinking. I believe one must maintain an open mind and remember that DNA repair and mutagenesis are far from being understood at the molecular level.

Pathak: Dr. Grossweiner, do you want to add any comments? I am calling on the molecular biologists first, and then we will go to the clinicians.

L. Grossweiner: I think that the working relationships among the photochemists, microbiologists, and physicians has been exemplary in this field, particularly because psoriasis is an intractable disease of unknown origin and apparently only contracted by humans. Nevertheless, with these handicaps, we have been able to devise an extremely effective therapy for psoriasis on an admittedly incomplete but valid molecular basis that has been followed from in vitro experiments up through microorganisms, laboratory animals, and humans. I think that this cooperation between the various photobiologists working at different levels should be strongly encouraged and that the problems that were addressed at this Conference will be partially solved in the next few years.

Pathak: Dr. Young, do you want to give us your opinion about psoralens and UVA?

A. Young: I just have a general comment about psoralens. I think it is useless, at least from the medical–clinical perspective, to study psoralens in great depth if they are not going to be clinically effective. If the psoralen is not going to be used by clinicians or if the general clinical opinion is that the drug is ineffective, then I believe, as a clinician, doing a lot of research on that psoralen is unjustifiable. Perhaps from other points of view one could justify it. However, I think we have to establish which compounds are likely to be useful clinically.

Pathak: Would a molecular biologist wish to add to this dicussion? Dr. Kraemer?

K. Kraemer: I would like to point out that what Dr. Smith has said with regard to variation in repair in bacteria probably holds true even more so with humans. We are just beginning to scratch the surface of the variation among humans with different diseases and sensitivities. Probably, many different repair systems are involved. If we can find a defect in 1 person, then that means that that system is working properly in the normal person.

D. M. Carter: One aspect of this field has not been stressed that has to do with the problems we experience when intro-

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ABBREVIATIONS: TMP = 4,5',8-trimethylpsoralen; 8-MOP = 8-methoxypsoralen; UVA = UV radiation at 320–400 nm; PUVA = psoralen plus UVA.

[1] Moderated by Madhu A. Pathak at the Conference on Photobiologic, Toxicologic, and Pharmacologic Aspects of Psoralens held in Research Triangle Park, North Carolina, March 1–3, 1982.

[2] *Address reprint requests to:* Madhu A. Pathak, M.B., Ph.D., Department of Dermatology, Massachusetts General Hospital, Warren 5, Room 566, Boston, Massachusetts 02114.

ducing new therapy into medical practice. There must be cooperation between the scientific community, the medical community, the industrial community, and the regulatory agencies in getting a product into use that has some reasonable possibility of success. The modality must be tested adequately if we are to guard against harming the public, but because we are a profit-making society, testing requirements must be feasible if they are to be performed by the drug companies.

We would be unwise if we did not acknowledge these problems that bear on the opportunities for clinical research because the regulations for testing, as valid as they may be, are sometimes restrictive in the development and use of new technology. The history of PUVA treatment is a good case in point.

Young: I have another general point that I would like to make. Frequently, we hear about comparisons of UVA doses. One group says it takes a certain number of joules to clear something and another group maintains that it takes a different number. However, to my knowledge, most of the dosimeters used in PUVA therapy have a narrow band of sensitivity which peaks in the 365-nm region. It may be that the 365-nm part of the spectrum is much less effective than the shorter UVA which is emitted by these lamps. So we may have a problem if we are not actually comparing biologically effective doses but comparing physical doses instead. That is just a general cautioning point which I think tends to be ignored. We still do not know the action spectrum for clearing psoriasis with PUVA. Most investigators who have done spectrum studies with PUVA suggest that clearing could well be accomplished with the shorter UVA wavelengths. Although these shorter wavelengths are not in vast quantities in the spectra output of the various lamps that are used, they could have a relatively high biologic effect.

E. Christophers: After PUVA therapy was introduced in Europe in 1974, many patients were given this therapy, and, after a short while, an adverse reaction was seen in many countries alerting the physicians that this treatment is carcinogenic and dangerous. Consequently, many patients were treated with UV radiation at 290–320 nm. One of the results of this Conference, I think, is that convincing evidence has been presented that this type of therapy is less dangerous than the continuous application of UVB, which is now used in many places.

Pathak: I now open the discussion to the clinical group. Dr. Parrish, this is your opportunity to tell us about the future of PUVA.

J. A. Parrish: I would like to thank Dr. Pathak again for his participation in organizing this meeting. I was sitting here thinking that he was probably going to call my name. I was thinking of three "Cs" and three "Ds." The first of the three "Cs" that I think the psoralen people need is *creative thinking.* I think we need to get ourselves free from some of the experimental pathways we have been exploring for a long time and do some creative thinking. The second "C" would be *critical evaluation.* We need to judge ourselves critically, and that is not always done. The third "C" would be *constant dialogue.* This meeting is a good example of that kind of dialogue.

The three "Ds" that I have been thinking of are: 1) I think we need to *diminish* ego investment in various kinds of thinking and treatment; keep a more open mind. 2) We need to *distrust* sweeping generalities. I think one of the problems we are struggling with is how monoadducts or diadducts function. Which of them is more mutagenic, carcinogenic, or therapeutic? I think that whenever we indulge in these generalities we are constantly knocked to our knees by the complex biologic situations that we study. So I think we need to distrust these sweeping simplifications. The third "D" I think is for *diligence*: fewer meetings so we can stay home and work for a while.

Pathak: Dr. Fitzpatrick, it is time for you to enrich us with some of your wisdom.

T. B. Fitzpatrick: Despite Dr. Parrish's distrust of generalities I am going to make a sweeping statement. I think we have a most difficult problem going forward from here. The ideal psoralen is nonerythemogenic, clinically effective, and noncarcinogenic. Finding an ideal one is probably achievable, but I do not know about the last characteristic, i.e., whether the carcinogenic one is achievable. As Dr. Carter brought up, we are in a "catch-22" (frustrating) situation because in our country and certainly more so in Europe, we are required to perform chronic toxicity studies before we can test substances in humans. Dr. Young questioned why develop a drug if it will not be used, but it cannot be used clinically unless 3 months of chronic toxicity research has been done, hence the catch-22.

Personally, as you all know, I am a hopeless optimist. I think we can overcome this and find a nonerythemogenic, noncarcinogenic, effective psoralen. Then, too, we have the retinoids which may prove useful for short-term therapy. We may also extend the number of joules we gave initially for a lifetime.

Unlike Dr. Parrish, I think this meeting was overdue, but we should not have another one for 5 to 10 years. We had not had a meeting since 1959, so we needed one. It has been tremendously helpful to have participants from such varied fields assembled to exchange ideas.

Smith: Maybe you will think differently when I say that whenever you have an agent that damages DNA you run the risk of cancer. If you are looking for something that is not going to cause cancer, then it must be a substance that does not damage DNA; this probably means it will not kill cells, and, therefore, it will be ineffective. All therapeutic agents that I know of are toxic; therefore, you will always be faced with risk–benefit analysis.

K. Wolff: I want to extend what I alluded to in my talk. You mentioned the dogmas. We have one dogma in dermatology and it concerns the disease psoriasis. The dogma in psoriasis is hyperproliferation. We have no evidence that the initial lesion has anything to do with hyperproliferation. There is inflammation, and this is my point. Perhaps we do not need a photosensitizer that interferes with DNA. This, of course, would exclude the psoralens. We would have to rename this Conference.

K. M. Halprin: I am not certain that every substance that interacts with DNA must cause cancer. If it is not repaired, it will not cause cancer. Possibly we can just destroy these cells. Either they are dead or they are undamaged, which would be an alternative.

Smith: From my understanding of radiation biology, that will not be the case.

Pathak: Dr. Smith, as a molecular biologist, you know there are ways to inhibit enzymes that synthesize DNA and bypass the system to effect the sequencing of the nucleotides, so we could use these approaches to tackle the problem. I can argue with this. That does not mean you are indicating that the DNA that is affected causes a cancerous condition.

Smith: I meant that if you damage DNA, lesions are also being repaired. There is no simple way to kill a cell with a nonrepairable lesion.

Pathak: At this meeting, you have witnessed the recent advances in the specialties of photomedicine, phototherapy, photochemistry, and photobiology. These advances are based on molecular concepts of quantum efficiency for selective delivery of photons (with defined wavelengths and defined UV doses) and their known interactions with important biomolecules, such as DNA, RNA, proteins, enzymes, oxygen, etc. In recent years, psoralens have been used by photobiologists, geneticists, biochemists, and clinicians as useful probes for basic research studies in the understanding of DNA damage, cell membrane damage, and DNA repair. Scientific reasoning and creative use of physical, chemical, and optical principles of photophysics, photochemistry, and photobiology can now be extended to treat human diseases successfully. Those who have seen severe cases of psoriasis, vitiligo, mycosis fungoides, etc. know and appreciate the meaning of these new advances. Until recently, we were using UV and visible radiation as a useful probe to study the mechanisms of skin photosensitization reactions. This basic knowledge has enabled us to use phototherapy and photochemotherapy successfully in the treatment of human diseases. I have no doubts that this successful approach is based on scientific reasoning complemented by the combination of critical, collaborative teamwork and the use of technological advances, both in the delivery of radiation and the interaction of the drug with the abnormal metabolic or proliferative condition of skin diseases.

Our task now is to control some of the harmful reactions of PUVA and continue our basic and clinical studies on 1) the mechanism of action of PUVA at the DNA and cell membrane levels (repair, cell transformation, etc.) and 2) the development of new techniques to modify the light-induced phototoxic (cell membrane damaging effects) and photocarcinogenic effects of PUVA, so that higher levels of safety will be assured without therapeutic effectiveness being sacrificed. I believe that photons, like bullets, can be delivered to the target area with minimal cellular damage and maximal therapeutic effects.

I want you all to know that you can send us your suggestions, and we will be glad to include them for future studies. Dr. June Dunnick will have some closing remarks, and then we will adjourn this meeting.

J. Dunnick: The stimulating discussions and presentation of ideas have made this a most interesting meeting. The National Toxicology Program has been delighted to have participants from foreign countries including France, West Germany, Italy, Finland, Austria, Denmark, and the United Kingdom. We are also pleased to have had participants from 14 states for a total of 96.

The National Toxicology Program has sponsored investigations in psoralen treatment for several years based on recommendations from the National Cancer Institute and the Food and Drug Administration. Initially, these 2 agencies were particularly interested in 8-MOP because it was the psoralen primarily used in the United States.

Many scientists from the National Toxicology Program have participated in the psoralen studies, and I would especially like to acknowledge the contributions that have come from our Program personnel: Dr. Ernest E. McConnell in pathology, Dr. C. W. Jameson in chemistry, Dr. Joseph Haseman in statistics, and Dr. John Moore in toxicology. When Dr. Thomas Orme was with the National Toxicology Program, he was instrumental in helping to set up the contract program in psoralen research.

The critical application of PUVA therapy in various fields is evident. However, continued comparison of animal and clinical data will yield useful information. Some of the toxicologic studies which we have planned and some which will be done by many of you and others throughout the world will help to elucidate the separate effects of psoralen and UV light from the combined effects.

The animal studies will emphasize the effects of these compounds on cancer development of the skin as well as throughout the body. Data from the animal experiments will enable us to predict what will occur in humans. Does PUVA therapy initiate or promote common or unusual tumors? What is the relationship between clinical results and interactions of psoralens with DNA?

The personnel in the National Toxicology Program have a broad interest in the problems of skin toxicity and compounds that interact with DNA. This Conference has served as a focal point for those interested in psoriasis and the use of PUVA for the treatment of other diseases. However, the discussions at this Conference have broader applications than the usefulness of PUVA alone. From a toxicologic standpoint, we can use these studies as models for UV light and chemical interactions.

I want to thank each of you for your presentations and contributions; I think everyone has done an outstanding job.

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